Regulation of the Sodium-Proton-Exchanger NHE3 and the Sodium-Phosphate-Cotransporter NaPi-IIa by PKA and EPAC

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1. Summary

The type IIa Na/Pi-cotransporter (NaPi-IIa; SLC34A1) is expressed in the brush border membrane (BBM) of renal proximal tubules where it mediates the bulk of P_i reabsorption from the primary urine. The Na/H-exchanger 3 (NHE3; SLC9A3) is also expressed in the BBM of proximal tubules and plays a major role in acid-base and extracellular volume regulation. Both NaPi-IIa and NHE3 are regulated by parathyroid hormone (PTH) and dopamine (DA). In the case of NaPi-IIa, both hormones induce membrane retrieval and lysosomal degradation. In contrast, NHE3 activity is inhibited in an early phase without reduction of the transporter expression in the BBM.

PTH and DA receptors belong to the superfamily of G-protein coupled receptors (GPCR) and signal via activation of adenylyl cyclase (AC) and phospholipase C (PLC). Activation of AC with the subsequent increase in intracellular cAMP plays a key role in the regulation of both NaPi-IIa and NHE3. Intracellular cAMP signals through two independent pathways: the cAMP-dependent protein kinase A (PKA) and the guanine exchange protein directly activated by cAMP (EPAC).

The aim of this study was to investigate the contribution of the PKA- and the EPAC-dependent pathways in the regulation of NaPi-IIa and NHE3. For that purpose, the activity of NaPi-IIa and NHE3 was determined in the absence/presence of several cAMP analogs which preferentially activate either the PKA or EPAC pathway. Studies were performed in Opossum kidney (OK) cells and in slices from mouse kidney. OK cells express endogenous NHE3 and NaPi-IIa transporters that are properly (apically) expressed and regulated. On the other hand, our lab has shown that slices from mouse kidney retain their morphological and functional properties within the time frame in which the effects of the different analogs were investigated. NHE3 activity was determined in OK cells by measurements of Na^+-dependent intracellular pH recovery rates, and in BBMV (isolated from slices of mouse kidney) by acridine-orange fluorimetry. NaPi-IIa activity was determined in OK cells by measurement of Na^+-dependent ^{32}P-uptake.

Activation of either the PKA or EPAC pathway resulted in inhibition of NHE3 activity, both in OK cells and kidney slices. The EPAC-induced effect was independent of PKA activation, as indicated by direct and indirect measurements of PKA activity. The PKA and EPAC effects were independent of activation of phospholipase C (PLC), as PLC activity was affected by neither activator. However, the PKA- as well as the EPAC-induced inhibition of NHE3 was sensitive to a MEK1/2-specific inhibitor, suggesting the implication of ERK1/2 in that process. Both PKA and EPAC inhibited NHE3 activity without inducing changes in the expression of the transporter in the BBM, suggesting that inhibition may involve modification of surface expressed transporters. On another hand, activation of PKA, but not of EPAC, inhibited NaPi-IIa activity in OK cells. The PKA-induced inhibition was mediated by endocytosis.

In summary, these results suggest that EPAC activation may represent a novel mechanism involved in the cAMP regulation of NHE3, whereas regulation of NaPi-IIa is mediated by PKA only.

2. Zusammenfassung

Der Typ IIa Natrium-abhängige Phosphatkotransporter (NaPi-IIa; SLC34A1) wird in der Bürstensaummembran der proximalen Tubuluszellen in der Niere exprimiert, wo der grösste Teil der Reabsorption von Phosphat aus dem Primärharn stattfindet. Der Natrium-Protonen-Austauscher 3 (NHE3, SLC9A3) ist ebenfalls in der Bürstensaummembran der proximalen Tubuluszellen lokalisiert und spielt eine wichtige Rolle im Säure/Base-Haushalt und in der Regulierung des extrazellulären Volumens. Sowohl NaPi-IIa als auch NHE3 werden über das Parathormon (PTH) und Dopamin (DA) reguliert. Beide Hormone induzieren die Internalisierung von NaPi-IIa, welches anschliessend in Lysosomen degradiert wird. Im Gegensatz dazu wird die Aktivität von NHE3 zunächst inhibiert, während die Expression in der Membran gleich bleibt.

PTH- und DA-Rezeptoren gehören zur Familie der G-Protein gekoppelten Rezeptoren und leiten Signale über die Aktivierung der Adenylatzyklase (AC) und der Phospholipase C (PLC) weiter. Die Aktivierung der AC und der dadurch ausgelöste Anstieg von intrazellulärem cAMP spielt eine wichtige Rolle in der Regulation von NaPi-IIa und NHE3. Da durch intrazelluläres cAMP zwei unabhängige Signalwege aktiviert werden: Die cAMP-abhängige Proteinkinase A (PKA) und das durch cAMP direkt aktivierte Guanin-Austauscher-Protein (EPAC).

Das Ziel dieser Arbeit war die Untersuchung der PKA- und EPAC-abhängigen Signalkaskaden und deren Rolle in der Regulierung von NaPi-IIa und NHE3. Zu diesem Zweck wurden verschieden modifizierte cAMP-Analoge verwendet, welche entweder PKA oder EPAC aktivieren. Die Aktivität von NaPi-IIa und NHE3 wurde in Gegenwart und Abwesenheit dieser modifizierten cAMP untersucht. Die Experimente wurden in kultivierten Opossumnierenzellen (OK-Zellen) und in Mausnierenschnitten durchgeführt. OK-Zellen exprimieren endogen NaPi-IIa- und NHE3-Proteine, welche vollständig in der Membran lokalisiert (apikal) sind und reguliert werden. Andererseits hat unser Labor gezeigt, dass Mausnierenschnitte die morphologischen und funktionalen Eigenschaften innerhalb des Zeitfensters, in welchem die verschiedenen modifizierten cAMP-Analoge untersucht wurden, beibehielten. Die Aktivität von NHE3 wurde mittels Messung der Aenderung der Na^+-abhängigen intrazellulären Alkalisierungsrate in OK-Zellen bestimmt. In Bürstensaummembranvesikeln (isoliert aus Mausnierenschnitten) wurde die Aktivität von NHE3 mittels Acridin-orange-Fluorimetrie ermittelt. Die Aktivität von NaPi-IIa wurde durch Messung der Na^+-abhängigen ^{32}P-Aufnahme bestimmt.

Die Aktivierung des PKA- oder EPAC-Signalweges resultierte sowohl in OK-Zellen als auch in Nierenschnitten in einer Inhibition der NHE3-Aktivität. Durch direkte und indirekte Messungen der PKA-Aktivität wurde gezeigt, dass der durch EPAC induzierte Effekt unabhängig von der PKA-Aktivierung war. Desweiteren waren die Effekte von PKA und EPAC unabhängig von Phospholipase C (PLC) Aktivierung, da die PLC-Aktivität durch keinen der beiden Aktivatoren (PKA und EPAC) beeinflusst wurde. Dennoch waren sowohl die PKA- als auch die EPAC-induzierte Inhibition von NHE3 sensitiv gegenüber einem MEK1/2-spezifischen Inhibitor. Dies impliziert eine Rolle von ERK1/2 in diesem Prozess. Sowohl PKA als auch EPAC inhibierten die Aktivität von NHE3, ohne dass sich die Expression

des Transporters in der Membran änderte. Andererseits führte PKA-Aktivierung, aber nicht EPAC-Aktivierung, zur Inhibition der NaPi-IIa-Aktivität in OK-Zellen. Diese Inhibition erfolgte über Endozytose.

Zusammenfassend deuten diese Resultate darauf hin, dass die Aktivierung von EPAC einen neuen Mechanismus darstellt, der an der Regulierung von NHE3 beteiligt ist. Die Regulierung von NaPi-IIa ist hingegen nur über PKA gesteuert.

3. Introduction

3. 1 The kidney

A Anatomy

The kidneys lie retroperitoneally in the back of the abdominal cavity on either side of the vertebral column. They are drained by the ureters, which carry the urine to the urinary bladder. Each kidney (Fig.1) is divided into two parts: an outer cortex and an inner medulla. The cortex contains glomeruli, convoluted tubules, cortical collecting ducts and associated blood vessels. The medulla (outer and inner medulla) is striated which results from the parallel arrangement of loops of Henle, medullary collecting ducts and blood vessels. The kidney is divided into about a dozen lobes, each consisting of a pyramid of medullary tissue plus the cortical tissue overlying its base and covering its sides. The apex of a medullary pyramid forms a renal papilla, which drains the urine into a minor calyx. Minor calices unite to form a major calyx, which in turn lead to the expanded renal pelvis drained by the ureter.

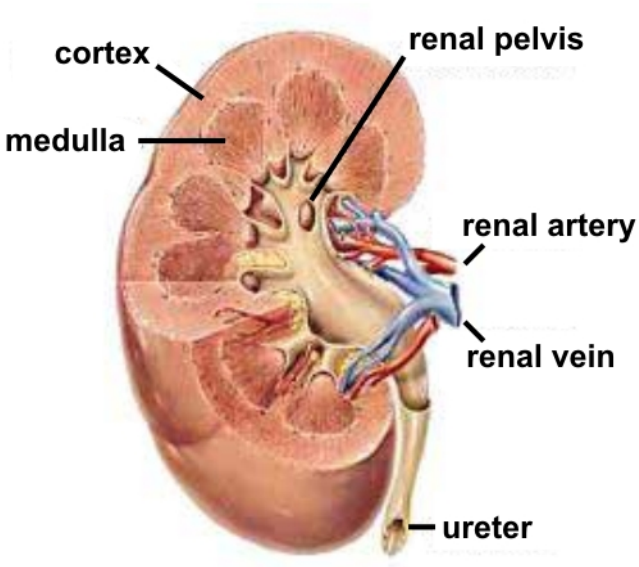

Figure 1: Kidney
Anatomical regions of the kidney.

B Structure of the nephron

The basic structural unit of the kidney is the nephron. Each human kidney contains about one million nephrons, each of them consisting of a glomerulus and a tubule. The glomerulus is a tuft of capillaries surrounded by the Bowman's capsule, whereas the tubule is an epithelial structure (Fig. 2). Because of the high hydrostatic pressure in the glomerular capillaries, the filtrate is forced out of the capillaries into the urinary space of the Bowman's capsule (ultrafiltration). The glomerular capillary wall allows unselective passage of small molecules (<10 kDa). From the urinary space of the Bowman's capsule, the filtrate reaches the lumen of the renal proximal tubule of the nephron where most of the valuable components of the filtrate (water, salt and metabolites) are returned to the blood.

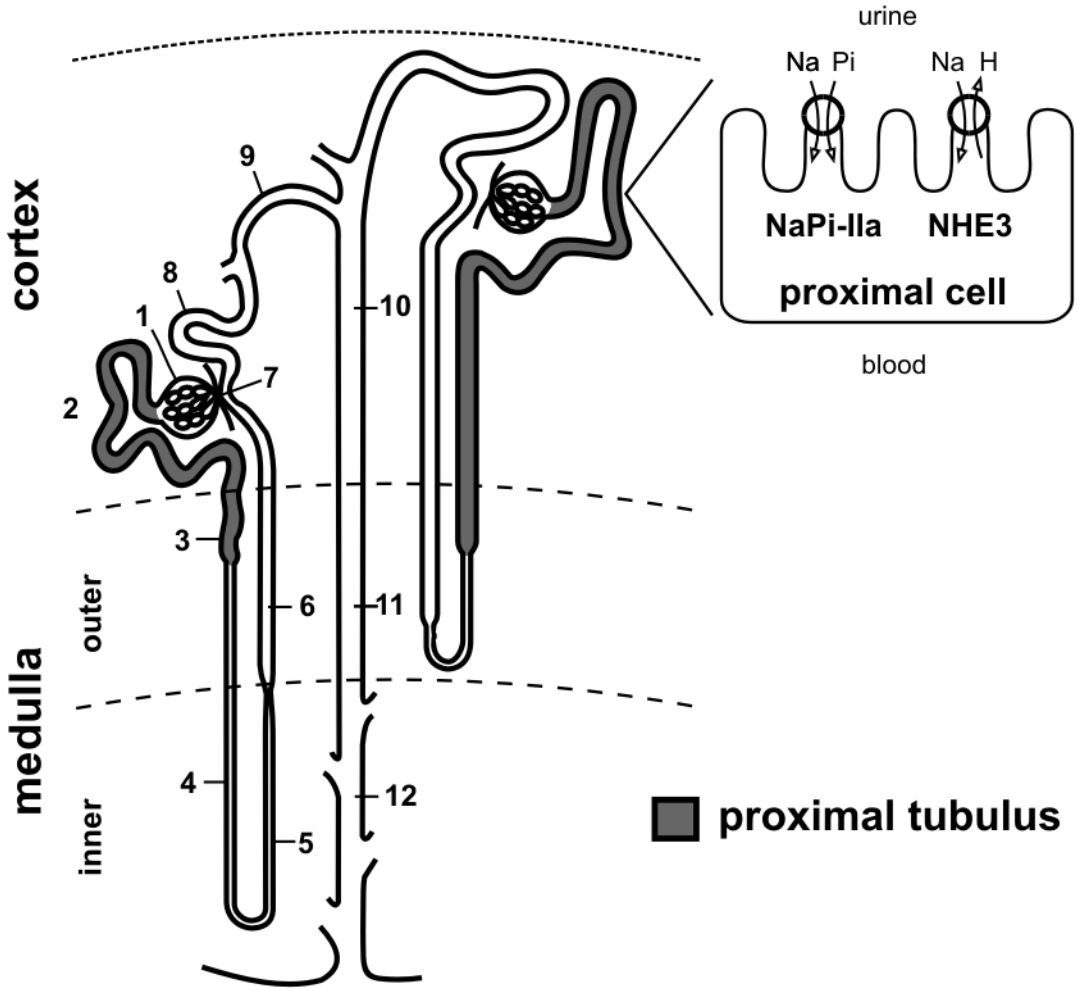

Figure 2: Nephron

Right: superficial nephron; left: juxtamedullary nephron. Shading depicts the expression pattern of NaPi-IIa and NHE3 along the nephron.

1: glomerulus, 2: proximal convoluted tubule, 3: proximal straight tubule, 4: thin descending limb, 5: thin ascending limb, 6: distal straight tubule (thick ascending limb), 7: macula densa, 8: distal convoluted tubule, 9: connecting tubule, 10: cortical collecting duct, 11: outer medullary collecting duct, 12: inner medullary collecting duct.

The renal tubule is divided into several anatomically and functionally defined segments. The first segment, the proximal tubule (PT), is divided into two parts: the proximal convoluted tubule, which coils and twists (convolutes) in the neighbourhood of its glomerulus, and the proximal straight tubule, which descends toward the renal medulla. The apical side of proximal epithelial cells contains a large number of extensions, or microvilli, which function is to increase the surface and therefore the rate of reabsorption. This specialized apical membrane is called brush border membrane (BBM). The PT is followed by the thin descending limb and thin ascending limb of Henle and the thick ascending limb of Henle. The length of the thin limb of the loop of Henle is variable, depending on the nephron generation: juxtamedullary types have high filtration rates and long limbs whereas superficial types have short or absent thin limbs. After the distal convoluted tubule, connecting tubule, cortical collecting tubule and medullary collecting ducts, the final urine is delivered via the pelvis to the ureter (Greger 1999; Kriz 2000).

C Function

The kidneys play a dominant role in regulating electrolyte composition, acid-base balance and the volume of extracellular fluids (blood plasma, intestinal fluid and lymph). In addition, the kidneys eliminate metabolic waste products. To sustain a high filtration rate of the plasma, approximately 20% of the cardiac output is filtered by the kidneys, resulting in the production of 180 liters of primary urine per day. Glomerular filtration covers large quantities of mineral electrolytes, water and organic compound presented to the tubules. The bulk of the filtrate, more than 99% of valuable constituents (e.g. water, sodium, chloride, glucose and urea), is returned to the body by tubular reabsorption, resulting in the excretion of around 1.5 liter of urine per day (Greger 1999). As for any other epithelia, vectorial tubular reabsorption relays on the asymmetrical distribution of transporters in the apical (luminal) and basolateral sides of the tubular cells. Both membranes have a very different protein and lipid composition. This asymmetry is conserved by the presence of tight junctions which separate apical from basolateral membranes preventing their components from diffusing and mixing. This work focuses on two Na^+-dependent transporters that are specifically expressed in the BBM of proximal tubules: the type IIa Na/Pi cotransporter and the Na/H-exchanger 3, (see chapters 3.2 and 3.3).

3. 2 Inorganic phosphate homeostasis

A General considerations

Inorganic phosphate (P_i) is involved in a large number of biochemical processes and is a structural component of DNA, RNA, proteins and phospholipids. Because of its limited solubility in the presence of divalent cations, the concentration of free P_i is balanced in the low millimolar range in both intra- and extracellular fluids. Bacteria, yeast, plants and vertebrates have developed their own strategies to control P_i homeostasis, with different membrane transporters involved in this process (Werner, Dehmelt et al. 1998). Several organs play a crucial role in vertebrate P_i homeostasis. The major storage compartments for P_i in the body are the bones where 85% of inorganic phosphate is present, whereas 14% is found in cells of soft tissues. Only about 1% of P_i is found in extracellular fluids. In the blood, 30% of phosphate is present in the free form and 70% in the bound form (Seldin 2000).

Three families of Na^+-dependent P_i-cotransporters have been identified: family I, II and III, or NaPi-I (SLC17A), NaPi-II (SLC34) and NaPi-III (SLC 20). NaPi-I from rabbit was the first member of the NaPi-I familiy to be cloned. However, its physiological roles and functions are yet unknown. The NaPi-II family has been proved as the major regulator of P_i homeostasis and represents a main topic of this work. Therefore, its properties and mechanisms of regulation will be dicussed in detail in the following section. The members of the third protein family, NaPi-III, have been identified as retroviral receptors. The widespread expression of this family suggests that NaPi-III is involved in supplying the basic cellular metabolic needs for P_i (Werner, Dehmelt et al. 1998).

B The NaPi-II (SLC34) family

The NaPi-II family is subdivided into three types: type IIa (NaPi-IIa), type IIb (NaPi-IIb) and type IIc (NaPi-IIc) (Murer, Forster et al. 2004). These cotransporters are expressed in several tissues and play a major role in P_i homeostasis. In kidney (NaPi-IIa and NaPi-IIc) and small intestine (NaPi-IIb), these cotransporters are located at the apical side of epithelial cells and mediate the rate limiting entry step in transepithelial movement of P_i. Physiological and pathophysiological regulation of renal and small intestinal epithelial transport of P_i occurs through alterations in the abundance of the respective NaPi-II cotransporters (Murer, Forster et al. 2004). In a physiological environment, all NaPi-II family members transport exclusively P_i ions in an obligatory sodium-dependent manner. Of the three types, NaPi-IIa and NaPi-IIb operate in an electrogenic manner, whereas NaPi-IIc operates electroneutrally.

The major site of NaPi-IIa expression is the renal proximal tubule where it is localized at the BBM (Custer, Lotscher et al. 1994) (Fig. 3). Besides in the kidney, expression of this protein has been described in osteoclasts and neurons (Gupta, Guo et al. 1997; Hisano, Haga et al. 1997). NaPi-IIa was

identified by functional expression cloning using *X. laevis* oocytes and a rat and human kidney cDNA library (Magagnin, Werner et al. 1993). The cotransport mediated by NaPi-IIa involves the inward transfer of one net positive charge per transport cycle. Divalent P_i is transported together with 3 Na^+ ions (Murer, Forster et al. 2004).

NaPi-IIb was identified based on EST clones derived from lung tissue (Hilfiker, Hattenhauer et al. 1998). By immunofluorescence, NaPi-IIb was localized in BBM of enterocytes, in the apical pole of alveolar type II cells as well as in apical membranes of mammary secretory cells. In addition, expression of NaPi-IIb has also been found in various other tissues such as testis, bones, liver and prostate (Xu, Bai et al. 1999). At the primary sequence level, NaPi-IIb differs from NaPi-IIa mainly in the C-terminus which is rich in cysteine residues and longer by approximately 50 amino acids. The cotransport mediated by NaPi-IIb is also electrogenic with a likely stoichiometry of 3 Na^+: 1 P_i (Murer, Forster et al. 2004).

NaPi-IIc has been found exclusively in kidney where it is localized at the BBM of proximal tubules of deep nephrons (Segawa, Kaneko et al. 2002). Expression of NaPi-IIc is higher in weaning mice and decreases progressively with age (Segawa, Kaneko et al. 2002). The cotransport mediated by NaPi-IIc is electroneutral (Segawa, Kaneko et al. 2002; Bacconi, Virkki et al. 2005).

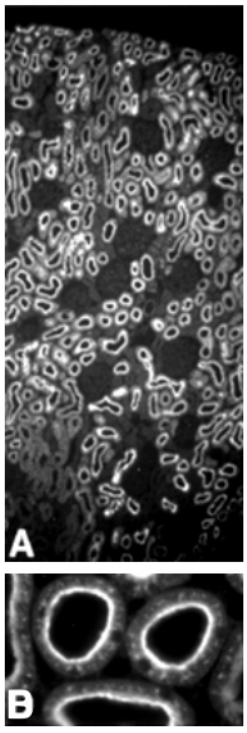

Figure 3: Immunohistochemistry of NaPi-IIa

Immunohistochemical detection of NaPi-IIa protein in kidneys. NaPi-Iia is specifically expressed in proximal tubules.
A) overview **B)** higher magnification. (Adapted from Traebert, Volkl et al. 2000).

C NaPi-IIa

NaPi-IIa consists of approximately 640 amino acids (Magagnin, Werner et al. 1993). Based on results obtained from hydropathy analysis, cysteine scanning mutagenesis and epitope-tagging studies, a model for the secondary topology of NaPi-IIa has been proposed (Lambert, Traebert et al. 1999) (Fig 4). The secondary structure predicts at least eight transmembrane domains (TMD1-8) with two re-entering loops between TMD2/TMD3 and TMD5/TMD6. Both the N- and C-termini are intracellular. A large extracellular loop (ECL-2) contains two N-glycosylation sites. An essential disulphide bridge in ECL-2 links each complementary part to form the functional unit. The re-entering loops (ICL-1 and ECL-3) are predicted to associate and form the transmembrane cotransport pathway (for review see (Murer, Forster et al. 2004). Due to the high homology between the three types of cotransporters, a similar topological model has also been proposed for NaPi-IIb and NaPi-IIc.

Renal expression of NaPi-IIa is restricted to the proximal tubule. By immunohistochemistry, it has been shown that NaPi-IIa is located exclusively in BBM and is uniformly distributed along the microvilli. Under normal conditions, NaPi-IIa is predominantly expressed in S1 segments of juxtamedullary nephrons (Custer, Lotscher et al. 1994). The Na^+/P_i-cotransport in BBM isolated from NaPi-IIa knockout mice is reduced by 70%, indicating that most of proximal tubular P_i reabsorption takes place via NaPi-IIa (Beck, Karaplis et al. 1998). It is not known via which pathway the remaining 30% are transported, although NaPi-IIc may be partially responsible (Tenenhouse, Martel et al. 2003).

Several renal wasting disorders based on changes in the expression of NaPi-IIa have been described. Studies related to the inherited disorders XLH (X-linked hypophosphatemia) and ADHR (autosomal dominant hypophosphatemic rickets) and the acquired disorder OHO (oncogenic hypophosphatemic osteomalacia) revealed that two proteins, namely PHEX and FGF23, play important roles in determining the abundance of NaPi-IIa in renal proximal tubules. Two naturally occurring variants of NaPi-IIa have been identified and linked to two cases of renal wasting disorders of phosphate (Prie, Huart et al. 2002). However, it appears that these variants are rather functionally normal polymorphisms than mutants (Virkki, Forster et al. 2003).

Proper apical expression of NaPi-IIa requires an intact C-terminal tail (Karim-Jimenez, Hernando et al. 2001), which last three residues (TRL) represent a PDZ-binding domain. This suggests that apical sorting and/or stabilization of NaPi-IIa involves the interaction with PDZ-proteins. Yeast two-hybrid screenings identified several proteins that interact with NaPi-IIa in a PDZ-dependent manner (Gisler, Stagljar et al. 2001). One of these proteins was the Na^+/H^+ exchanger regulatory factor NHERF1 (see chapter D). Apical expression of NaPi-IIa was partially disrupted upon expression of a dominant negative NHERF1 construct in a proximal cell model (Opossum kidney cells), suggesting that binding to NHERF1 is required for proper apical localization of NaPi-IIa (Hernando, Deliot et al. 2002). This sorting and/or stabilization role was confirmed in a NHERF1 knock-out model. These animals were characterized by high urinary excretion of P_i as consequence of a reduced expression of NaPi-IIa in BBM (Shenolikar, Voltz et al. 2002).

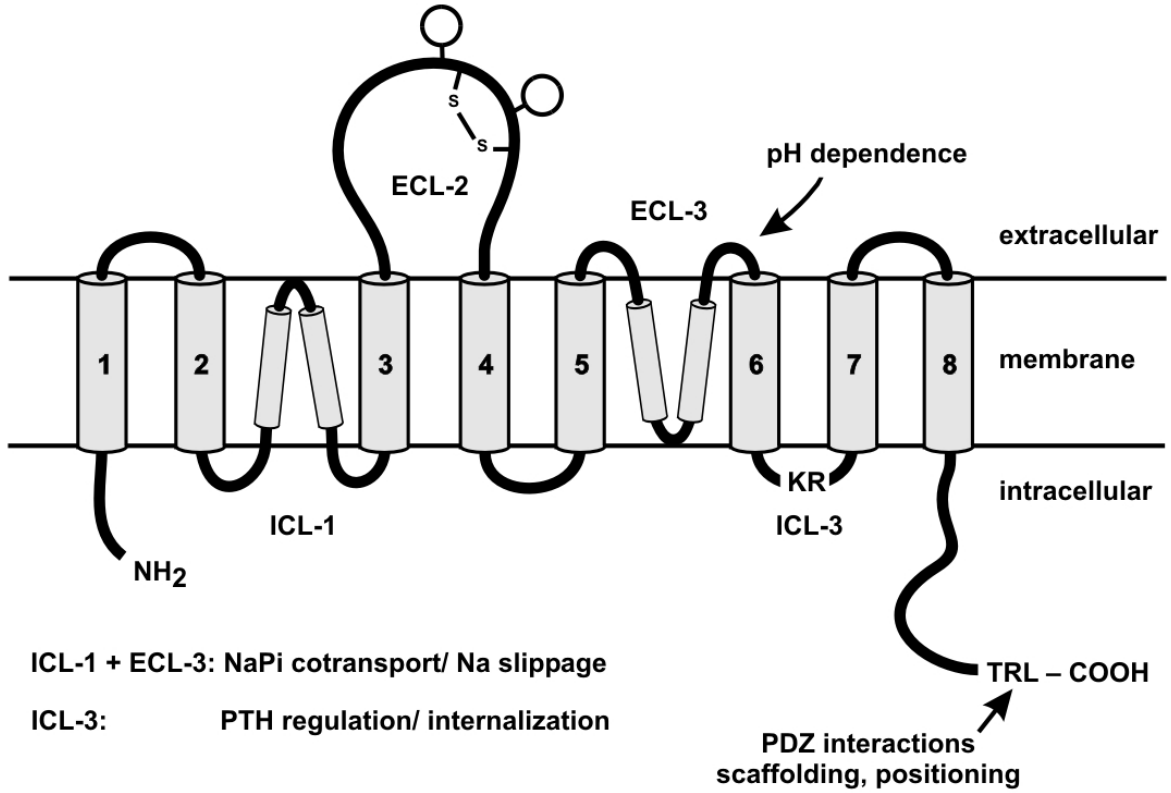

Figure 4: Secondary structure of NaPi-IIa

Both N- and C-termini are located intracellularly. The 2 glycosylation sites in ECL-2 are indicated by circles. The KR motif in the ICL-3 responsible for PTH sensitivity is indicated. Cysteine residues likely to form a disulfide bridge are depicted. ICL: intracellular loop; ECL: extracellular loop; 1-8: transmembrane regions; ICL-1 and ECL-3: re-entering loops.

D Regulation of NaPi-IIa

Renal P_i reabsorption is regulated by a variety of factors, including dietary P_i intake, metabolic acidosis, and several homones, such as parathyroid hormone (PTH), dopamine (DA) and growth factors. Several studies demonstrated that altered rates of cotransport correlated with an altered amount of cotransporters in the apical membrane (for review see Murer and Biber 1997; Murer, Hernando et al. 2000; Murer, Forster et al. 2004).

PTH represents one of the most powerful phosphaturic hormones. After PTH infusion, the rates of proximal P_i reabsorption in rats decreased due to a reduction of the amount of NaPi-IIa. Immunohistochemical studies demonstrated that after these treatments, NaPi-IIa cotransporters are transiently accumulated in the subapical compartment and are associated with small and large endocytic vacuoles (Kempson, Lotscher et al. 1995; Lotscher, Kaissling et al. 1996; Bacic, Le Hir et al.

2005). Internalization of NaPi-IIa most likely occurs at the invaginated intermicrovillar regions, possibly via clathrin-coated vesicles. An on-and-off switch at the cleft sites is postulated which specifically targets NaPi-IIa to clathrin-coated vesicles. Morphological and biochemical data suggest that internalized NaPi-IIa molecules do not reside in an intracellular compartment from which they could be recycled to the plasma membrane, but are instead routed directly to the lysosomes (Keusch, Traebert et al. 1998). Similar observations were made also with OK cells where PTH led to a complete disappearance of NaPi-IIa, which could be prevented by the addition of inhibitors of lysosomal degradation (Pfister, Ruf et al. 1998). Thus, the PTH inhibition of proximal P_i reabsorption goes via internalization of type II cotransporters and represents an irreversible process.

The signaling pathways leading to PTH-induced internalization of NaPi-IIa and subsequent lysosomal degradation have been investigated in some detail (Fig. 5). PTH can bind to either apical or basolateral receptors. PTH receptors belong to the class II G-protein coupled receptor (GPCR) family (Abou-Samra, Juppner et al. 1992). They can couple to heterotrimeric G-proteins of the G_s, $G_{i/o}$ or G_q family (see section 3.4). Activation of both apical and basolateral PTH receptors leads to endocytosis and lysosomal degradation of NaPi-IIa. However, and despite the fact that both sides of the membrane express identical receptors, each process is mediated by different signaling pathways. Studies in isolated proximal tubules indicated that NaPi-IIa downregulation upon binding of PTH to apical receptors preferentially involves activation of the phospholipase C (PLC)/protein kinase C (PKC) cascade. In contrast, downregulation of NaPi-IIa upon binding of PTH to basolateral receptors is preferentially mediated by the adenylate cyclase (AC)/protein kinase A (PKA) pathway (Traebert, Volkl et al. 2000). This different signaling/coupling is mediated, at least partially, by the specific expression of NHERF1 in the apical membrane of proximal tubules. NHERF1, which interacts with the C-terminal tail of NaPi-IIa, binds simultaneously to PTH receptors and to PLC. Therefore, the presence of NHERF1 in the BBM directs the receptor signaling via the PLC/PKC pathway. Consistently with this scheme, treatment with 3-34 PTH (a fragment that only activates PLC) on kidney slices from NHERF1 knockout mice does not lead to activation of PLC, neither to downregulation of NaPi-IIa (Capuano et al., in preparation). In the absence of NHERF1 (basolateral membranes) the PTH-induced inhibition would be coupled to AC/PKA (Gisler, Stagljar et al. 2001; Gisler, Madjdpour et al. 2003; Gisler, Pribanic et al. 2003) (Fig. 5). Both the apical and basolateral pathways converge, at least partially, at the level of ERK1/2, although the steps that connect ERK1/2 activation with NaPi-IIa endocytosis remain unknown (Bacic, Schulz et al. 2003).

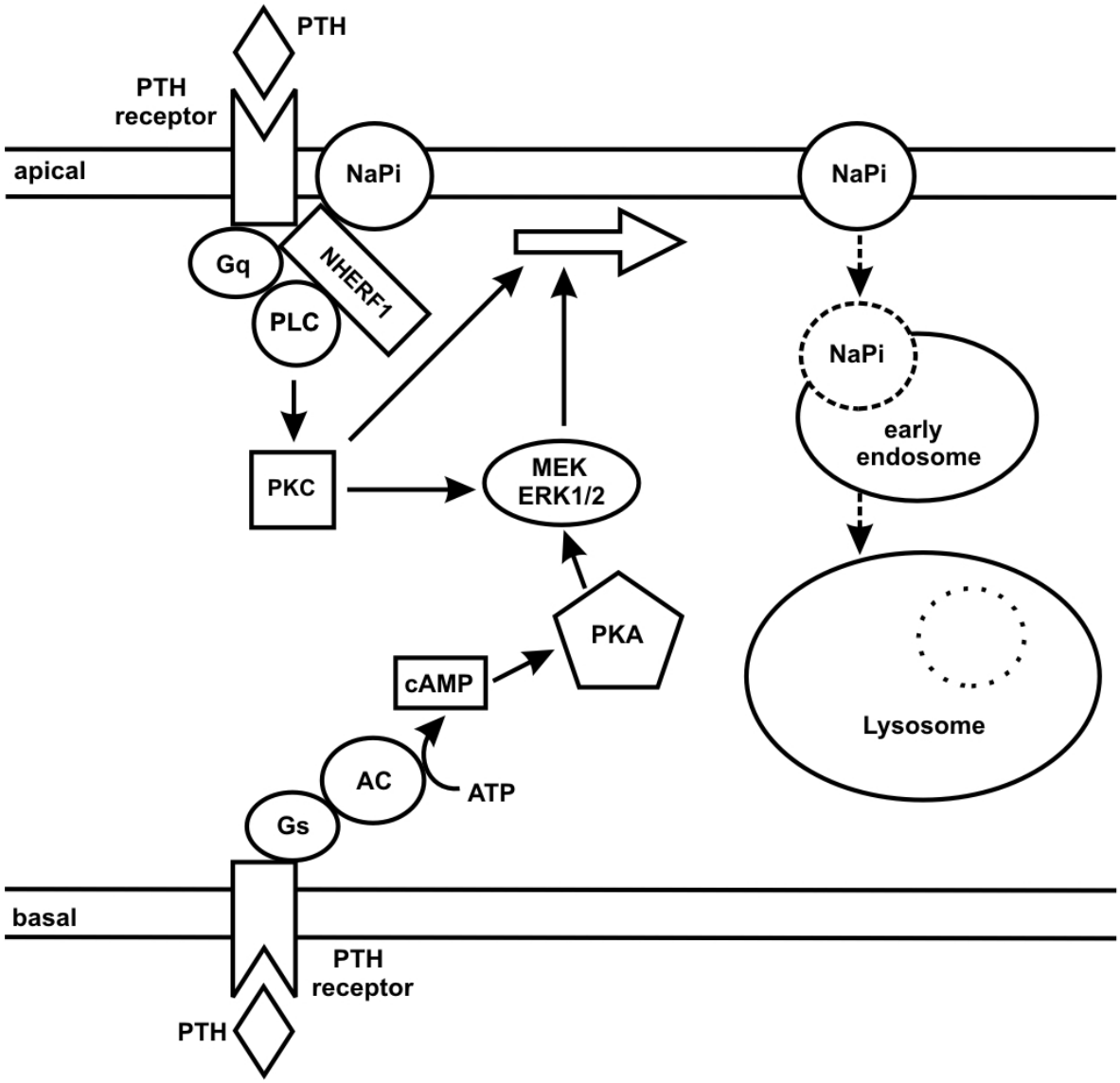

Figure 5: PTH signaling leading to NaPi-IIa downregulation

Basal PTH receptors are coupled to Gs and activate PKA via cAMP, whereas apical PTH receptors are coupled to Gq via NHERF1 and activate PKC via DAG. NaPi-IIa is endocytosed and degraded in the lysosome.

The identification of molecular domains within NaPi-IIa that are required for the PTH-induced downregulation was possible due to the PTH insensitivity of NaPi-IIb. Thus, the analysis of the PTH responsiveness of different NaPi-IIa/NaPi-IIb chimeras led to the finding that the last intracellular loop (ICL-3) was required for the PTH response. In particular, two conserved residues, positively charged in NaPi-IIa (KR) and non-charged in NaPi-IIb (NI), determine the sensitivity or insensitivity to PTH (Fig. 4)

(Karim-Jimenez, Hernando et al. 2000). The ICL-3 was found to interact, in a KR dependent manner, with PEX19, a farnesylated protein involved in peroxisomal biosynthesis. Overexpression of PEX19 induces endocytosis of NaPi-IIa even in the absence of downregulatory stimuli (Ito, Iidawa et al. 2004).

Similar to PTH, also DA induces internalization and degradation of NaPi-IIa. As the PTH receptors, the DA receptors belong to the class II of GPCR. So far, five different DA receptors have been cloned (Sibley and Monsma 1992). Based on their association with heterotrimetric G-proteins they are classified into two families: the D1-like receptors are coupled to G_s and therefore they signal through increases of intracellular cyclic adenosine 3', 5'-monophosphate (cAMP), whereas the D2-like receptors couple to G_i and signal through decreases of intracellular cAMP. Proximal tubules expressed both types of receptors in the apical as well as in the basolateral membrane. Although the effect of DA on NaPi-IIa has not been analyzed as thoroughly as the effect of PTH, recent studies indicate that DA-induced downregulation of the cotransporters is mediated via luminal D1-like receptors (Bacic, Capuano et al. 2005).

With regard to the factors that induce increases of proximal tubule P_i reabsorption, the most dramatic effect is observed shortly after a low-P_i diet. Four hours after treatment, expression of NaPi-IIa increased close to the maximum level that is observed after prolonged (days) adaptation to a low P_i-diet. Upregulation of NaPi-IIa in response to a low P_i-diet depends on *de novo* synthesis. This effect is partially prevented in NHERF1 knockout mice (Weinman, Boddeti et al. 2003; Cunningham, E et al. 2005). Studies concerning depletion of PTH have so far been performed mostly in cell culture (OK cells) and indicated that upon removal of PTH the recovery (upregulation) of NaPi-IIa was also completely dependent on protein *de novo* synthesis (Pfister, Lederer et al. 1997).

3. 3 pH regulation

A General considerations

A basic property of life is the ability of an organism to regulate cellular pH, volume and ion composition. Cellular pH is crucial for biological functions such as cell proliferation, drug resistance and apoptosis. Four major types of pH regulators have been identified: the vacuolar proton pump, the sodium-proton exchangers, the bicarbonate transporters and the monocarboxylate transporters (Izumi, Torigoe et al. 2003).

a) The vacuolar proton pump is a multisubunit protein that pumps H^+ from the cytoplasm to the lumen of vacuoles or into the extracellular space, using the energy produced by ATP hydrolysis (Wagner, Finberg et al. 2004).

b) The sodium/proton exchanger (NHE) family (SLC9) consists of secondary active transporters that mediate an electroneutral exchange of Na^+ for H^+ (Brett, Donowitz et al. 2005). The sodium/proton exchanger 3 (NHE3) represents a major topic in this work; therefore, the properties of the NHE family will be discussed in detail below.

c) The bicarbonate transporter (BCT) superfamily is categorized into members of the solute carrier 4 (SLC4) and members of the solute carrier 26 (SLC26) family. The SLC4 family contains electroneutral ion exchangers (AE1-AE4) as well as electroneutral (NDCBE, NCBE and NBCnl) or electrogenic (NBCE1, NBCE2) Na^+/HCO_3^- cotransporters. The SLC26 family contains 10 members that function as anion exchangers, of which SLC26A3 (DRA), SLC26A4 (Pendrin), SL26A6 (CFEX) and SLC26A7 also mediate Cl^-/HCO_3^- exchange (Mount and Romero 2004).

d) The family of monocarboxylate transporters (MCT, SLC16) transports monocarboxylates, such as lactate, coupled to H^+. Lactate is the end-product of glycolysis, therefore its efflux has to be controlled in order to prevent cellular acidification (Halestrap and Meredith 2004).

B The NHE (SLC9) family

Transmembrane exchange of H^+ for Na^+ is ubiquitous in organisms across all phyla and kingdoms and underlies fundamental homeostatic mechanisms to control these ions. In mammals, the family of sodium/proton exchangers (NHE) catalyzes the electroneutral exchange of Na^+ and H^+ down the Na^+-gradient. These exchangers are crucial for numerous physiological processes, ranging from the fine control of intracellular pH and cell volume to systemic electrolytes, acid-base and fluid volume homeostasis. NHE activity also facilitates the progression of other cellular events such as adhesion, migration and proliferation.

Thus far, 8 distinct NHE genes (NHE1/SLC9A1 to NHE8/SLC9A8) have been identified in the human genome. The genes encode proteins of various primary sequence identity (25-70%), but share a common predicted secondary structure comprising 12 conserved membrane-spanning segments at the amino-terminus and a more divergent, cytoplasmically-oriented, carboxy-terminus (Fig. 6). They show considerable heterogeneity in their patterns of tissue/cell expression and membrane localization (Orlowski and Grinstein 2004; Brett, Donowitz et al. 2005).

By comparison, the source of energy that drives mammalian NHEs at the plasma membrane differs from their bacterial counterparts. Instead of an electrochemical H^+ gradient, it is the inward Na^+ gradient (established by plasma membrane Na^+/K^+-ATPase pumps) that drives the countertransport of H^+ in an electroneutral manner.

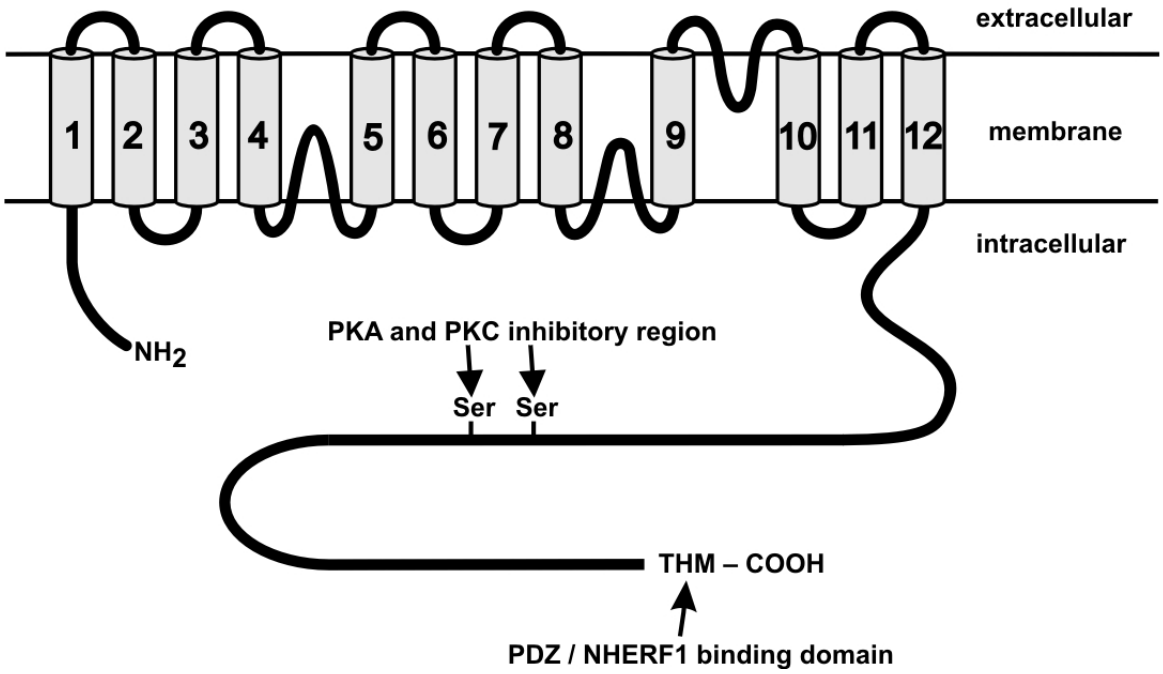

Figure 6: Secondary structure of NHE3

Both N- and C-termini are located intracellularly. The long C-terminus contains the indicated interaction sites. 1-12: transmembrane regions.

C NHE3

The sodium/proton exchanger 3 (NHE3) is expressed predominantly in epithelia of the kidney and the gastrointestinal tract, but is also detected at low levels in other tissues. NHE3 is localized in the luminal membranes of discrete nephron and intestinal segments. In the kidney, it is located predominantly in the proximal tubule and, to a lesser extent, in the medullary thick ascending limb of Henle (Fig. 7). In addition to the microvillus, NHE3 is detected in a discrete population of clathrin-associated subapical endosomes, where it could serve as a reservoir of functional transporters that shuttle to and from the BBM in response to hormones (Chow, Khurana et al. 1999). NHE3 also contributes to the acute pH regulation of the endomembrane compartment, a process that facilitates receptor-mediated endocytosis (Gekle, Drumm et al. 1999). NHE3 is a major contributor to the bulk of Na^+ and fluid reabsorption by the proximal tubule. The associated secretion of H^+ by NHE3 into the lumen of renal tubules is also essential for approximately two-thirds of renal HCO_3^- reabsorption. NHE3 regulates basal as well as meal-stimulated ileal Na^+ absorption *in vivo*. NHE3-null mice exhibit massive diarrhea and alkalinization

of the intestinal luminal contents, sharply decreased HCO_3^- and fluid absorption in proximal convoluted tubules, mild acidosis, reduced blood pressure, elevated serum aldosterone and higher renal renin mRNA expression, consistent with a volume-contracted state of the animals (Schultheis, Clarke et al. 1998).

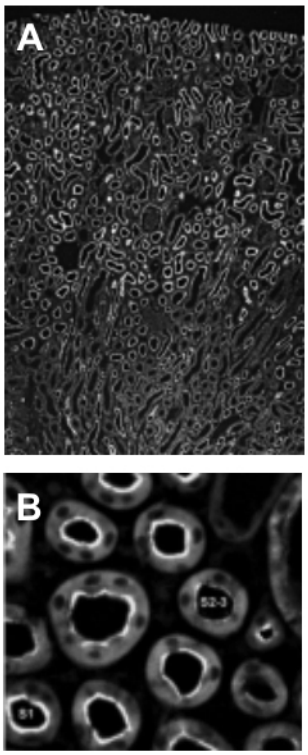

Figure 7: NHE3 immunodetection in kidney cortex
Immunohistochemical detection of NHE3 protein.
A) overview **B)** higher magnification. (Adapted from Bacic, Kaissling et al. 2003).

D Regulation of NHE3

Several studies have provided evidence for a modulation of a dynamic equilibrium between surface and endomembrane NHE3 transporters. NHE3 undergoes constitutive uptake into clathrin-coated vesicles (Chow, Khurana et al. 1999) and is recycled back to the plasma membrane in a phosphatidylinositol 3-kinase (PI3K)- dependent manner (Kurashima, Szabo et al. 1998). A variety of hormones and physical parameters, such as osmolality, influence NHE3 function, thereby contributing to the fine control of electrolyte and fluid homeostasis (Orlowski and Grinstein 2004). Some of these factors, including α-adrenergic receptor agonists, endothelin, and growth factors (epidermal growth factor and fibroblast

growth factor), stimulate NHE3. In contrast, hormones activating cAMP-dependent PKA, such as PTH and DA, reduce renal Na^+ and HCO_3^- reabsorption in part by inhibiting apical NHE3 activity.

Inhibition of NHE3 in renal cells by cAMP-elevating PTH does not parallel the course of disappearance of the exchanger (Collazo, Fan et al. 2000). Instead, this inhibition is due to the reduction of the transporter's apparent affinity for H^+ as consequence of phosphorylation of serine residues (Moe, Amemiya et al. 1995; Kurashima, Yu et al. 1997; Zhao, Wiederkehr et al. 1999; Collazo, Fan et al. 2000). Only upon long exposure times (>2 h) there is also a reduction of its maximum velocity, reflecting a decrease of the number of transporters expressed in the BBM (Fig. 8) (Hayashi, Szaszi et al. 2002). Therefore, PTH and DA inhibit both NaPi-IIa and NHE3, although by different mechanisms, as will be discussed below. Classical experiments with BBM indicated the presence of a cofactor required for the cAMP-induced inhibition of NHE3. These studies led to the identification of the Na/H-exchanger regulatory factors NHERF1 and NHERF2 (Fig. 8) (Shenolikar and Weinman 2001). In mouse, both NHERF isoforms are expressed in the BBM of proximal tubules. They contain two PDZ domains that mediate specific protein-proteins interactions and thereby serve as adaptors (Fig. 8). They also have a MERM (merlin/ ezrin/ radixin/ moesin)-binding domain that mediates binding to cytoskeletal proteins. In addition to its role as cytoskeletal linker, ezrin is also a PKA-anchoring protein (AKAP) (Weinman, Steplock et al. 2000). The present model suggests that NHERF1, through its ability to bind ezrin, mediates the scaffolding of NHE3 and PKA. Upon activation of its catalytic activity (in response to increases in intracellular cAMP), PKA inhibits NHE3 activity by phosphorylation of several serine residues located in the cytoplasmic domain (Moe, Amemiya et al. 1995; Kurashima, Yu et al. 1997; Zhao, Wiederkehr et al. 1999; Collazo, Fan et al. 2000). Consistent with this model, cAMP failed to regulate NHE3 activity in the BBM isolated from NHERF1 knockout mice (Weinman, Steplock et al. 2003). However, and unlike NaPi-IIa, the expression of NHE3 in the BBM of NHERF1 knockouts was normal, suggesting that NHERF1 is required for regulation, but not for apical expression of the transporter.

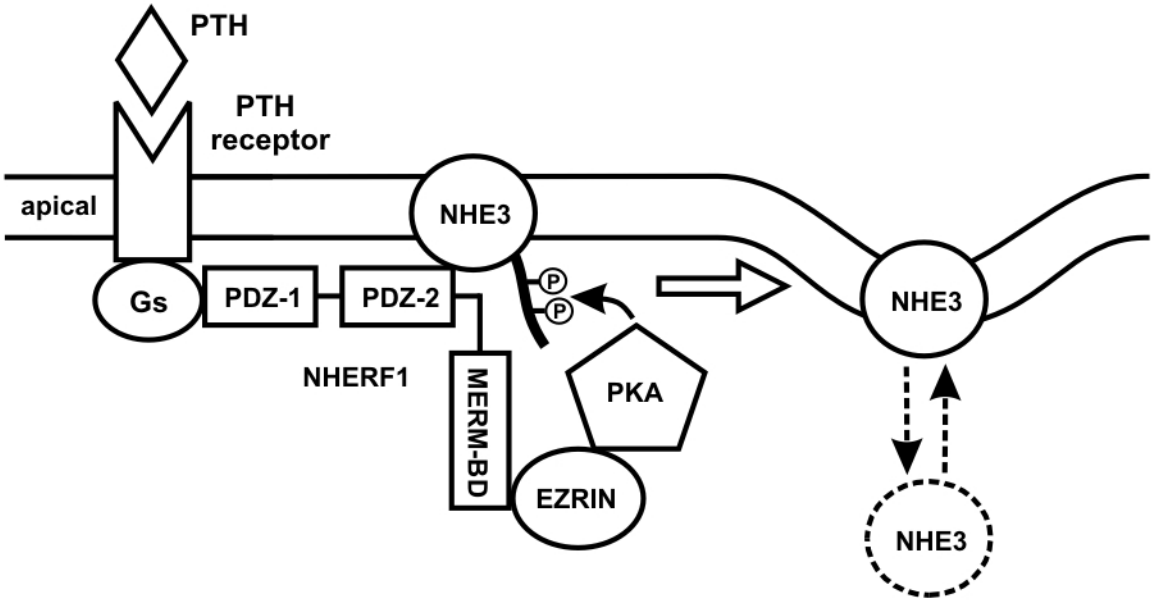

Figure 8: PTH signaling leading to NHE3 inhibition

Apical PTH receptors coupled to PKA via G_s and NHERF1 lead to inhibition of NHE3. NHERF1 binds through its MERM binding domain (MERM-BD) to EZRIN, which is coupled to PKA, and through its second PDZ domain (PDZ-2) to NHE3. PKA inhibits NHE3 via phosphorylation of serines. NHE3 is endocytosed in a later phase, and endocytosed molecules can recycle back to the membrane.

3. 4 Cell signaling

A GPCR: signaling via cAMP

As mentioned above, PTH and DA are two potent regulators of NaPi-IIa and NHE3 activity. Both hormones act via G-protein coupled receptors (GPCR) expressed in renal proximal tubules. With more than 1000 members, the family of GPCR represents the largest group of cell surface receptors encoded by the mammalian genome (Gutkind 2000). Based on partial homologies of their most conserved domains, they are classified as class I, II and III. PTH and DA receptors belong to the class II. In all cases, receptor signaling involves interaction with heterotrimeric G-proteins.

Heterotrimeric G-proteins contain three subunits referred as Gα, Gβ and Gγ. So far, about 20 Gα, 5 Gβ and 16 Gγ subunits have been identified in mammals. According to the homology of the Gα subunit, the G-proteins are divided into four families: G_s, G_i, G_q, G_{12} (reviewed in (Gutkind 2000). Upon ligand binding, the GPCRs undergo a conformational change that increases their affinity for the Gα subunits. The interaction with the receptors promotes the release of guanosine diphosphate (GDP) from the Gα subunit and its exchange for guanosine triphosphate (GTP). In the GTP-bound form, the

Gαβγ heterotrimer dissociates into a Gα monomer and a Gβγ dimer. Then, the activated Gα (GTP-bound) and the Gβγ initiate intracellular signaling cascades by acting on a variety of effector molecules. Gα subunits regulate the activity of several second messenger-generating systems. G_s and G_i signal through activation and inhibition of adenylyl cyclase (AC), respectively, therefore modulating the intracellular cAMP levels. G_q couple to phospholipase C (PLC) and modulate the concentration of diacylglycerol (DAG) (Fig. 9). In addition to the Gα subunits, also the Gβγ dimers have a role in intracellular signaling; thus, dimers from the G_i family are also reported to activate PLC.

PTH and D1-dopamine receptors couple to G_s and activate the cAMP signaling pathway. In addition, both receptors activate the PLC cascade by coupling either to G_q or G_i (Abou-Samra, Juppner et al. 1992; Sibley and Monsma 1992).

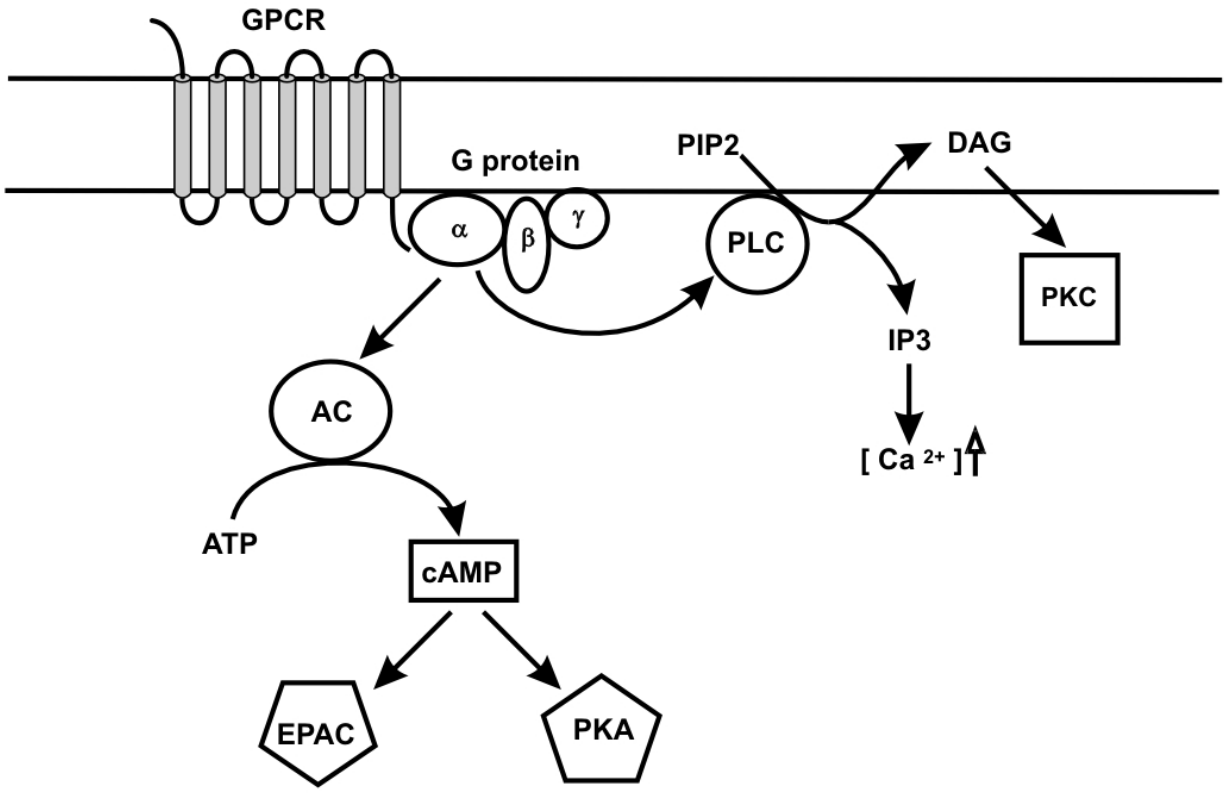

Figure 9: GPCR signaling

GPCR signaling can either lead to activation of adenylyl cyclase (AC) or phospholipase C (PLC). Activation of PLC leads to the production of DAG and IP3 which activate PKC and increase intracellular Ca^{2+}, respectively. Activation of AC induces an increase of intracellular cAMP which in turn can activate either PKA or EPAC.

B Protein kinase A (PKA)

The cAMP-dependent protein kinase A (PKA) pathway is one of the most common and versatile signaling pathways in eukaryotic cells and is involved in the regulation of cellular functions in almost all tissues in mammals. cAMP was the first identified second messenger. The intracellular level of cAMP is regulated by the balance between the activity of two types of enzymes: adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE) (Fimia and Sassone-Corsi 2001). Various extracellular signals converge in this signaling pathway through ligand binding to GPCR. The cAMP/PKA pathway must therefore be tightly regulated at several levels to maintain specificity in the multitude of signal inputs (Tasken and Aandahl 2004).

PKA is a heterotetramer composed of two regulatory (R) and two catalytic (C) subunits. Both the regulatory (RIα, RIβ, RIIα, RIIβ) and the catalytic (Cα, Cβ, Cγ) subunits possess distinct physical and biological properties, are differently expressed and able to form different isoforms of PKA holoenzymes. Binding of four cAMP molecules (two to each R subunit) leads to a conformational change and dissociation into an R-subunit-dimer (four cAMP molecules bound) and two C-monomers. The C-subunits then become catalytically active and phosphorylate serine and threonine residues on specific substrate proteins (Tasken, Hansson et al. 2000; Tasken and Aandahl 2004) (Fig. 10). Different PKA isozymes with distinct biochemical properties and cell-specific expression contribute to cell and organ specificity. PKA anchoring proteins (AKAP) target PKA to specific substrates and distinct subcellular compartments, providing spatial and temporal specificity for mediation of biological effects channelled through the cAMP/PKA pathway (Tasken and Aandahl 2004).

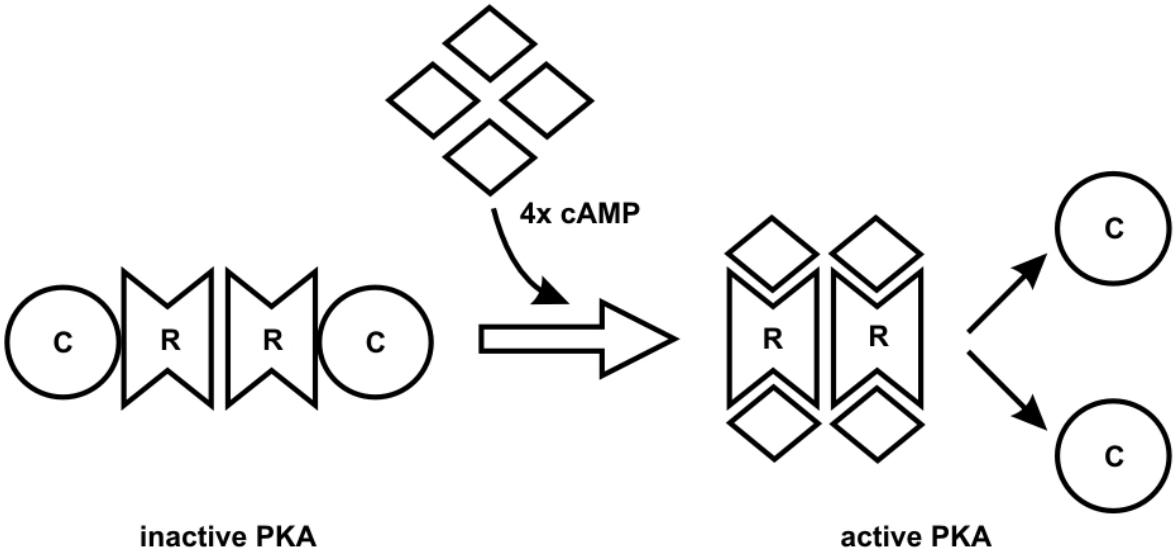

Figure 10: PKA activation

Four cAMP bind to the two regulatory (R) subunits which leads to dissociation of the two catalytic (C) subunits.

PKA has been regarded as the only effector of cAMP in most eukaryotic cells. However, the effect of cAMP on several cellular functions such as proliferation, gene expression or activation of protein kinase B (PKB/Akt) and mitogen-activated protein kinase (MAPK) has been shown to be cell specific, although the molecular mechanisms underlying this specificity are not fully understood. Recently, a new cAMP-dependent signaling pathway, independent of the classical PKA cascade, was discovered: the guanine exchange factor EPAC (exchange protein directly activated by cAMP).

C Exchange protein directly activated by cAMP (EPAC)

EPAC was identified when cAMP-induced activation of the Ras-like small GTPase Rap1 was found to be insensitive to inhibition of PKA (de Rooij, Zwartkruis et al. 1998). Although Rap1 is a substrate for PKA (Quilliam, Mueller et al. 1991), a mutated Rap1 lacking the PKA phosphorylation site is still activated by EPAC in response to cAMP (de Rooij, Zwartkruis et al. 1998). The presence of this alternative pathway may help to explain some of the cell specific cAMP responses.

EPAC1 and the closely related EPAC2 are proteins of 881 and 1011 residues, respectively (Kawasaki, Springett et al. 1998; Bos 2003). They catalyze, in a cAMP-dependent manner, the exchange of GTP for GDP that transforms Rap1 and Rap2 into an activated state (de Rooij, Zwartkruis et al. 1998). EPAC1 and EPAC2 contain, in addition to a cAMP-binding domain and a GEF (guanine nucleotide exchange factor) domain for Rap1 and Rap2, a DEP (Dishevelled, Egl-10, Pleckstrin) domain involved in membrane localization, and a REM (Ras-exchanger motif) that interacts with the GEF domain (de Rooij, Zwartkruis et al. 1998); for review see (Bos 2003) (Fig. 11). EPAC 2 contains a second cAMP-binding domain (A domain) at its amino terminus.

In the absence of cAMP, the catalytic activity of EPAC is inhibited by an intramolecular interaction between the GEF and the REM domains. Binding of cAMP promotes the disassembly of the inhibitory interaction, and EPAC becomes activated (Fig. 12). The A domain of EPAC2 binds cAMP with a lower affinity than both the cAMP-binding domain of EPAC1 and the B domain of EPAC2. EPAC1 and the B domain of EPAC2 bind cAMP *in vitro* with a K_m of about 40 μM, whereas the K_m of PKA is about 1 μM (de Rooij, Rehmann et al. 2000). This difference in affinity could be the consequence of differences in key amino acids in the cAMP-binding pocket and is suggested to allow EPAC to respond to changes in cAMP concentrations in a range in which PKA is already saturated (Springett, Kawasaki et al. 2004). The two isoforms of EPAC show a differential pattern of expression: EPAC1 mRNA has been detected in the thyroid, kidney, ovary, skeletal muscle and in specific regions of the brain, whereas EPAC2 expression is restricted to regions of the brain and the adrenal gland (Kawasaki, Springett et al. 1998; Laroche-Joubert, Marsy et al. 2002).

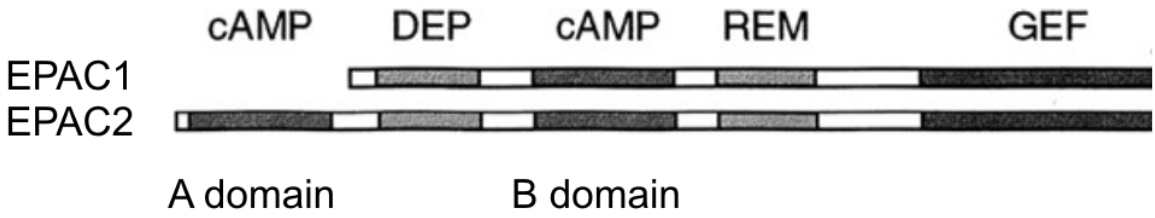

Figure 11: Structural domains of EPAC1 and EPAC2

cAMP: cAMP binding domain; DEP: Dishevelled, Egl-10, Pleckstrin domain; REM: Ras exchange motif; GEF: guanine nucleotide exchange factor.

The primary target of EPAC is Rap1, a member of the Ras family of small GTPases (de Rooij, Zwartkruis et al. 1998). Rap1 can bind to either GTP or GDP. The GTP-bound form (active form) associates with various effector molecules to transmit the signal, whereas the GDP-bound form (inactive form) does not (for review see (Kometani, Ishida et al. 2004) (Fig. 12). Therefore, the GTP/GDP transition acts as a molecular switch for signal transduction. Rap1 is activated by specific guanine nucleotide exchange factors (Rap1-GEFs), in response to several extracellular stimuli. These GEFs catalyze the dissociation of GDP and enable Rap1 to rebind to GTP. There are several types of Rap1-GEFs, among them EPAC1 and EPAC2, which induce exchange of GTP for GDP in response to increases in intracellular cAMP. The intrinsic GTPase activity of Rap1 is very low. This GTPase activity has an inhibitory effect on Rap1 signaling and is activated by specific GTPase-activating proteins (Rap1-GAPs). Therefore, signaling through Rap1 can be regulated by Rap1-GEFs and Rap1-GAPs. Effector molecules of Rap1 include Rac, Raf and Ral.

A role of EPAC1 and Rap1 in several cAMP-regulated processes, such as cell adhesion and migration (Bos 2003), insulin secretion (Nakazaki, Crane et al. 2002) and regulation of H^+/K^+-ATPase activity in kidney cortical collecting duct (Laroche-Joubert, Marsy et al. 2002) has been already demonstrated. Several intracellular cascades may mediate these effects. Thus, EPAC1 may regulate the extracellular signal regulated kinase ERK through activation of the Rap1/B-Raf pathway (Laroche-Joubert, Marsy et al. 2002), the PKB pathway through a Rap1-dependent activation of the phosphatidylinositol 3-kinase (PI3K) (Mei, Qiao et al. 2002) or the phospholipase C–ε (PLC-ε) pathway through Rap2B (Schmidt, Evellin et al. 2001). A brief description of these three cascades and the proposed cross-talk between EPAC and PKA is presented in the following sections.

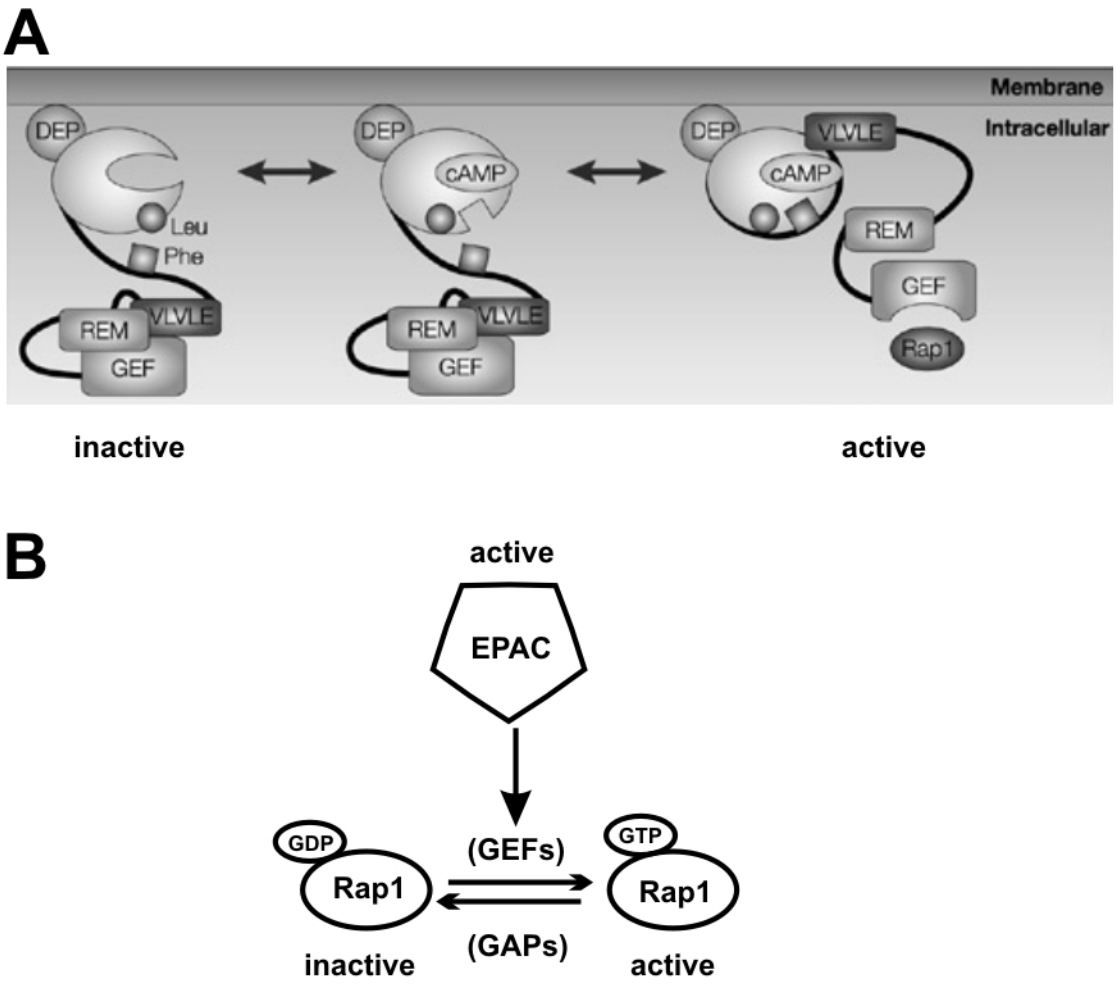

Figure 12: Activation of EPAC

A) A two-step model for EPAC activation. In step 1, on binding cAMP, a conserved leucine (Leu) in the phosphate-binding cassette (PBC) is displaced, which allows the hinge region that contains a conserved phenylalanine (Phe) to bend towards the cAMP-binding pocket. In step 2, the lid binds to the base of cAMP, which induces a conformational change that releases the inhibitory constraint on the catalytic GEF domain. REM: Ras-exchanger motif; GEF: guanine nucleotide exchange factor; DEP: Dishevelled, Egl-10, Pleckstrin domain; VLVLE: conserved valine-leucine-valine-leucine-glutamate sequence; GAP: GTPase activating protein. (Adapted from Bos 2003) **B)** active EPAC induces the exchange of GTP for GDP in Rap1.

D Phospholipase C (PLC)

As indicated above, binding of a hormone ligand to a GPCR results in activation and dissociation of a heterotrimeric G-protein into a GTP-bound α-monomer and a free $\beta\gamma$-dimer. The α_q-subunit as well as some $\beta\gamma$-dimers activate phospholipase C (PLC), which hydrolizes phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 raises the cytosolic Ca^{2+} concentration, while DAG activates the protein kinase C (PKC).

The PLC family comprises a diverse group of enzymes that differ in structure and tissue distribution. Eleven distinct isoforms of PLC have been identified in mammals and classified on the basis of amino acid sequence into 5 types: β, γ, δ, ε, ζ (Fig. 13A). All 5 types contain conserved catalytic X and Y domains and a Ca^{2+}-dependent lipid-binding C2-domain. Ca^{2+} is required for catalytic function. Structural and mutational studies have identified residues within the catalytic domain that likely contribute to substrate recognition, Ca^{2+} binding and catalysis (Rhee 2001). The 5 PLC types differ in the mechanisms by which they are activated (Rhee, Suh et al. 1989; Rhee 2001; Wing, Bourdon et al. 2003). PLC-β is activated by both α- and $\beta\gamma$-subunits of heterotrimeric G-proteins. PLC-γ contains Src homology (SH2 and SH3) domains and is activated by receptor or cytosolic tyrosine kinases. PLC-ε is of particular interest for this work since it has been shown to be regulated by EPAC in a Rap2B dependent manner (Fig. 13B). In addition , PLC-ε is directly regulated by Ras and represents a novel Ras effector (Kelley, Reks et al. 2001; Rhee 2001). The mechanisms of regulation of PLC-δ and PLC-ζ are less clear.

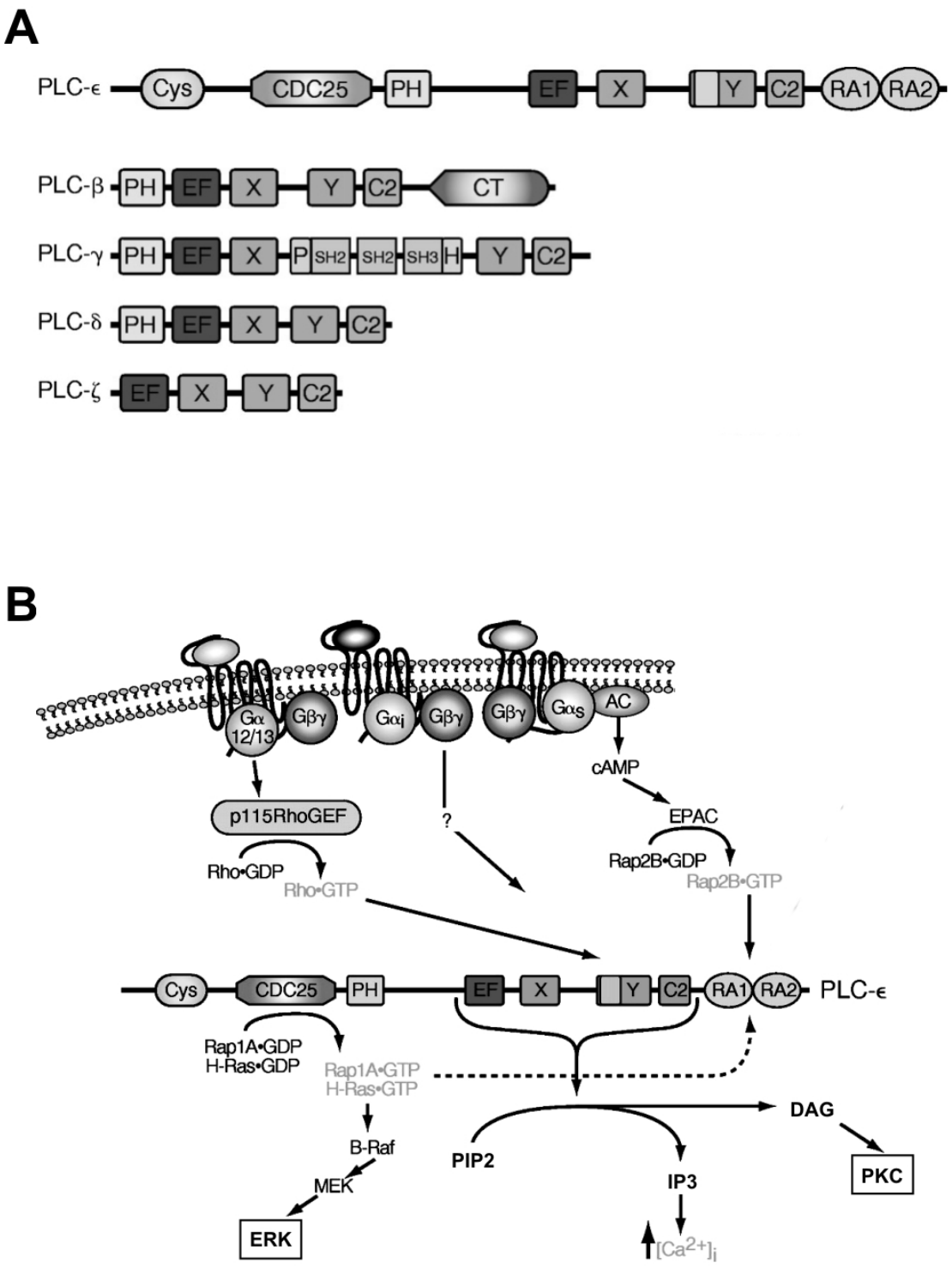

Figure 13: Phospholipase C (PLC)

A) Structural domains of the 5 types of PLC isozymes. Cys: cysteine-rich region; CDC25: guanine nucleotide exchange domain conserved among GEF; PH: pleckstrin homology domain; EF: EF-hand domain; X and Y: core catalytic domains; C2: Ca^{2+}/lipid binding domain; RA1 and RA2: Ras-associating domains; SH2 and SH3: Src homology domains. **B)** PLC-ε signaling pathways: PLC-ε is stimulated by $G\alpha_{12/13}$, Gβγ, and activated members of Ras and Rho small GTPases to yield downstream second messengers IP3 and DAG. In addition, ERK signaling pathways are propagated as a result of the guanine nucleotide exchange activity of the PLC-ε CDC25 domain. A positive feedback loop is schematized in which CDC25-promoted activation of H-Ras and/or Rap1A in turn activates the lipase activity of PLC-ε upon binding the C-terminal RA domains (dashed line). (Adapted from Wing, Bourdon et al. 2003)

E Protein kinase B (PKB/Akt)

Protein kinase B (PKB/Akt) has emerged as a central player in the signal transduction pathways activated in response to growth factors or insulin and is thought to contribute to several cellular functions including nutrient metabolism, cell growth, transcriptional regulation and cell survival (Brazil and Hemmings 2001). PKB/Akt is a serine/threonine kinase that belongs to the PKA/PKG/PKC (AGC) superfamily of protein kinases. Members of this superfamily share structural homology within their catalytic domain and have similar mechanisms of activation. Deregulations of some of these kinases are often associated with human diseases including cancer and diabetes. PKB/Akt was initially identified by 3 independent research groups, based on the homology to PKA and PKC or as the cellular homolog to the retroviral oncogene Akt.

In mammals, 3 PKB/Akt genes have been identified: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (reviewed in (Brazil and Hemmings 2001; Song, Ouyang et al. 2005). All 3 isoforms consist of a conserved domain structure: an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a carboxyl-terminal regulatory domain (Fig. 14). The PH domain interacts with membrane-bound lipids produced by phosphatidylinositol 3-kinase (PI3K), such as phoshatidylinositol-3,4,5-trisphosphate (PIP3). The kinase domain of PKB, located in the central region of the molecule, shares a high similarity with other AGC kinases such as PKA, PKC, p70S6K and p90RSK. All three PKB isoforms have a carboxyl terminal regulatory domain that contains the F-X-X-F/Y-S/T-Y/F hydrophobic motif (where F is phenylalanine, Y is tyrosine, S is serine, T is threonine and X is any amino acid) characteristic of the AGC kinase family. In all mammalian PKB isoforms, this motif is FPQFSY (where P is proline and Q is glutamine). Phosphorylation of the serine or threonine residue in this hydrophobic motif is necessary for full activation of the kinase. PKB is stimulated by numerous growth factors, cytokines, hormones and neurotransmitters in a PI3K-dependent manner (for review see (Hanada, Feng et al. 2004). These signals lead to activation of PI3K and generation of the membrane-bound PIP3. PIP3 then recruits PKB to the membrane, where it becomes phosphorylated at threonine 308 (in the kinase domain) and serine 473 (in the regulatory domain) by two upstream kinases (phosphoinositide-dependent kinase 1 (PDK1) and a yet to be identified serine kinase).

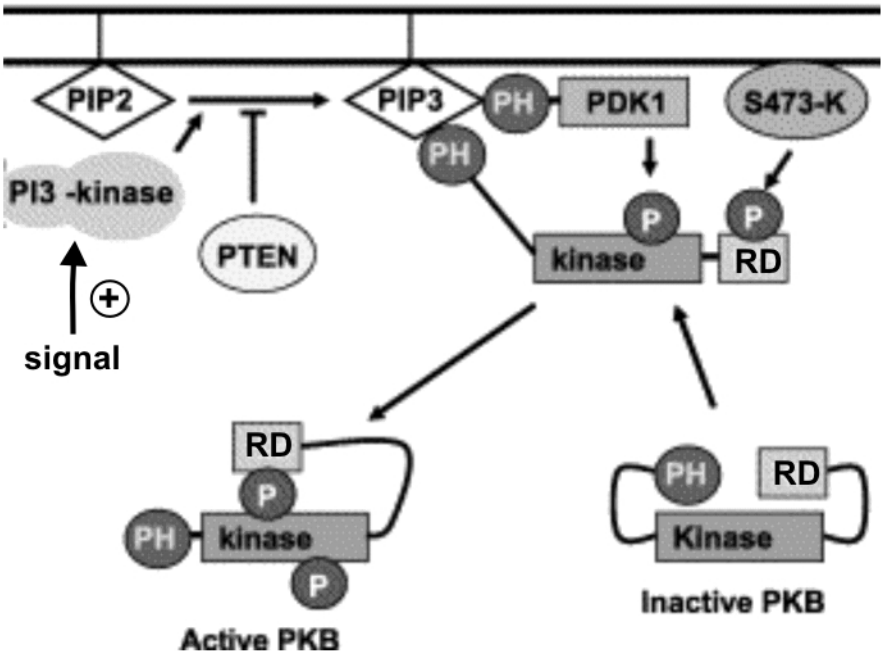

Figure 14: Schematic representation of PKB activation

Activated phosphatidylinositol 3-kinase (PI3K) produces PIP3 which organizes PKB, PDK1 and, probably, Ser 473-kinase on the plasma membrane, where PKB becomes phosphorylated and activated. Activated PKB then translocates from the cytosol to the nucleus. PTEN: protein phosphatese and tensin homolog deleted on chromosome 10; PDK1: phosphoinositide-dependent kinase 1; RD: regulatory domain; PH: pleckstrin homology domain. (Adapted from Hanada, Feng et al. 2004)

The PKB pathway exhibits cell type-specific responses to increases in intracellular cAMP. Both activation and inhibition of PKB have been reported upon PKA stimulation (Fig. 15). The PKA-induced activation seems to require phosphorylation of the threonine 308 located in the catalytic domain of PKB, a process that is independent of PI3K (Filippa, Sable et al. 1999). On the contrary, the PKA-induced inhibition takes place in a PI3K-dependent manner. Thus, PKA inhibits PKB by blocking the coupling between PKB and its upstream regulator PDK1, a process that requires PKA phosphorylation of Rap1b (Kim, Jee et al. 2001; Mei, Qiao et al. 2002). On another hand, EPAC has been reported to activate PKB via the PI3K pathway, most probably in a Rap1-dependent manner (Fig. 15) (Mei, Qiao et al. 2002). Interestingly, cAMP (via PKA) inhibits the PKB activity in wild type HEK293 cells, whereas PKB is activated upon cAMP treatment in HEK293 cells transfected with EPAC (Mei, Qiao et al. 2002).

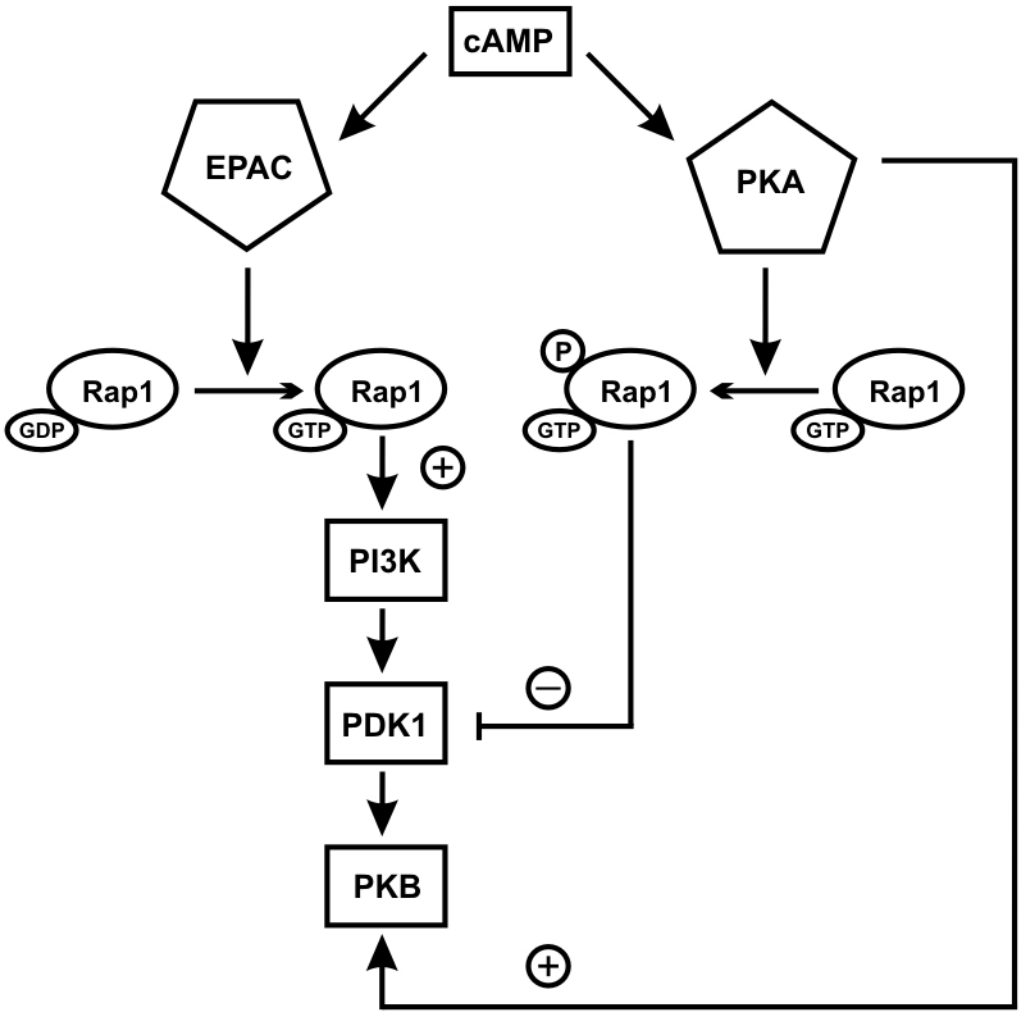

Figure 15: Mechanism of PKB regulation by cAMP

cAMP activates PKA or EPAC. EPAC leads to transformation of GDP-bound Rap1 to active GTP-bound Rap1. PKA phosphorylates Rap1 (GTP-bound). In response to the common activator cAMP, PKA and EPAC may mediate opposing effects on PKB activation. PI3K: phosphatidylinositol-3-kinase; PDK1: phosphoinositide-dependent kinase 1.

F MAPK: ERK 1/2

The mitogen-activated protein kinase (MAPK) family is affected by a number of GPCR (Gutkind 2000). MAPK control many cellular events from complex programs, such as embryogenesis, cell differentiation, cell proliferation, and cell death, to short-term changes required for homeostasis and acute hormonal responses (Chen, Gibson et al. 2001). The MAPK family can be stimulated not only by hormones, but also by growth factors, cytokines and even mechanical and environmental stress (Gutkind 2000).

So far, around 20 MAPKs have been identified in mammals. They can be subdivided into 4 families: extracellular signal-regulated kinases 1/2 (ERK1/2), jun NH_2-terminal kinase (JNK), p38 kinase (p38)

and ERK 5 family (Fig. 16). Although each MAPK has unique characteristics, a number of features are shared by all the MAPK pathways studied to date. Thus, they are activated by protein kinase cascades that contain at least two upstream kinases. The kinases immediately upstream are members of the MAP/ERK kinase (MEK) family. MAPK require tyrosine and threonine phosphorylation, both catalyzed by MEKs, to become highly active. As a consequence, these kinases are inactivated by all 3 major groups of protein phosphatases: those removing phosphate from serine/threonine or from tyrosine and the dual-specificity phosphatases which remove phosphate from both. MAPK phosphorylate serine or threonine residues most often followed by proline residues (Lewis, Shapiro et al. 1998). Following phosphorylation by their immediate upstream MEK, members of the MAPK familiy translocate to the nucleus where they phosphorylate transcription factors which regulate the expression of genes (Marinissen and Gutkind 2001). However, phosphorylation of cytoplasmic substrates has also been reported.

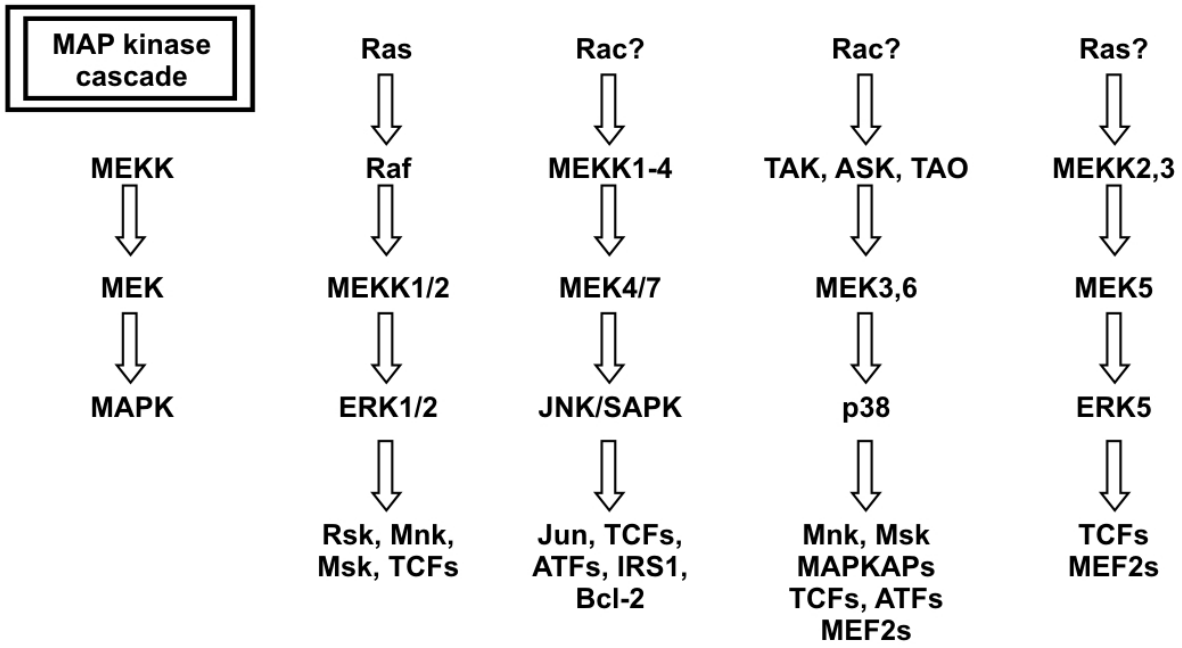

Figure 16: MAPK cascades

The left column shows a general MAPK cascade including a MAP/ERK kinase kinase, a MAP/ERK kinase and a MAPK.
ERK: extracellular signal-regulated kinase; MEK: MAP/ERK kinase; MEKK: MEK kinase; TCF: ternary complex factor; ATF; activating transcription factor; MEF: myocyte-enhancing factor; IRS1: insulin receptor substrate 1; Rsk: ribosomal S6 kinase; Mnk: MAP kinase-interacting kinase; Msk: mitogen- and stress-activated protein kinase; MAPKAP: MAP kinase-activated protein kinase. (Adapted from Chen, Gibson et al. 2001)

The ERK1/2 kinase cascade consists of a MAPK kinase kinase of the Raf family that phosphorylates and thereby activates downstream MAPK kinases (MKK1/2 or MEK1/2), which in turn phosphorylate

and activate ERK1/2 (Fig. 17) The two isoforms ERK1 (42 kDa) and ERK2 (44 kDa) are 83% identical, with most differences outside the kinase core (Boulton and Cobb 1991). Regulation of ERK1/2 by GPCR is highly complex and dependent on the cell type (Werry, Sexton et al. 2005). Both $G\alpha_s$-and $G\alpha_q$-coupled receptors can transduce signals to ERK1/2. Activation of ERK1/2 upon increases in intracellular cAMP induced by $G\alpha_s$-coupled receptors can be mediated by PKA or EPAC. The PKA response takes place via activation of the small GTPases Ras or Rap1, or via direct phosphorylation of B-Raf. EPAC-induced activation of ERK1/2 involves GTP-loading of Rap1, followed by activation of B-Raf (Fig. 17). ERK1/2 is activated in response to $G\alpha_q$-coupled receptors as a consequence of PKC-mediated phosphorylation of Raf. In this regard, recent studies from our lab have shown that ERK1/2 inhibitors fully prevent the endocytosis of NaPi-IIa induced by 8-Br-cAMP, whereas the response to PKC agonists is only partially prevented. Thus, NaPi-IIa downregulation induced upon activation of both PKA and PKC is mediated, at least partially, by activation of ERK1/2 (Bacic, Schulz et al. 2003). However, ERK1/2 inhibitors did not affect the dopamine-induced downregulation of the cotransporter (Bacic, Capuano et al. 2005). In addition, inhibition of ERK1/2 blocks the cAMP-induced stimulation of H^+, K^+-ATPase in kidney cells, an effect that has been attributed to EPAC (Laroche-Joubert, Marsy et al. 2002).

Both the ERK1/2 and PKB pathways lie downstream of the small GTPase Ras. Both signaling pathways are often simultaneously activated in response to growth factors and hormones. In some systems, Ras acts as an upstream positive effector of both the MAPK pathway and the PI3K/PKB pathway (Katz and McCormick 1997; Rommel and Hafen 1998). However, in myoblast cells (Rommel, Clarke et al. 1999), as well as in a breast cancer cell line (Zimmermann and Moelling 1999) PKB antagonizes the MAPK pathway by direct phosphorylation of Raf on a serine residue. Indeed, PKB has been described as a potent negative regulator of Raf-1 (Zimmermann and Moelling 1999). This cross-regulation depends on the state of differentiation of the cell: PKB activation inhibited the MAPK pathway in differentiated myotubes, but not in their myoblast precursors. The stage-specific inhibitory action of PKB correlates with its stage-specific ability to form a complex with Raf, suggesting the existence of differentially expressed mediators of an inhibitory PKB-Raf complex (Rommel, Clarke et al. 1999). Manipulation of these pathways during muscle differentiation indicated that inhibition of the MAPK pathway promoted differentiation, whereas inhibition of PI3K/PKB blocked differentiation (Bennett and Tonks 1997; Coolican, Samuel et al. 1997). Taken all these considerations together, it becomes clear that depending on the cell type, the same type of stimulation may trigger different sets of signaling cascades, thus leading to different, and often opposite, effects (Chiu, Ma et al. 2005)

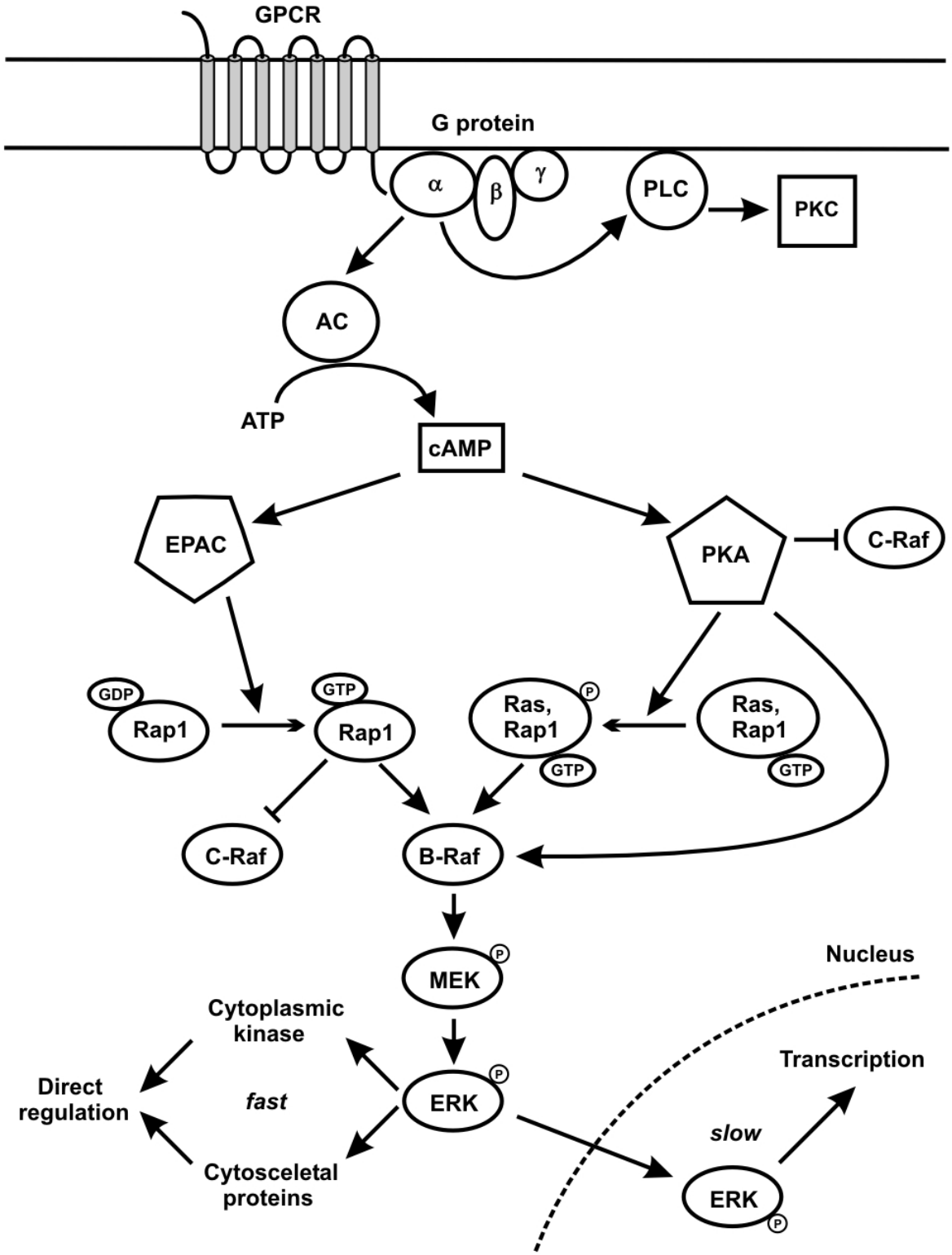

Figure 17: ERK signaling

cAMP activates either PKA or EPAC. EPAC leads to transformation of GDP-bound Rap1 to active GTP-bound Rap1. PKA phosphorylates Ras or Rap1. Rap1 or Ras activate B-Raf, which phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. ERK can activate transcription factors in the nucleus (slow) or lead to a direct activation via cytoplasmic kinases or cytosceletal proteins (fast).

4. Aim of the work

The aim of this study was to investigate the contribution of the protein kinase A (PKA) and the recently identified guanine exchange protein directly activated by cAMP (EPAC) pathways to the cAMP-induced inhibition of NHE3 and NaPi-IIa.

In the first part of this work, modified analogs of cAMP that preferentially activate either PKA (6-MB-cAMP) or EPAC (8-pCPT-2'-O-Me-cAMP) were applied in Opossum kidney (OK) cells and in murine kidney slices. The effect of PKA and EPAC activators on the activity and expression of both NHE3 and NaPi-IIa was investigated. Because these analogs preferentially, but not exclusively, activate either pathway, experiments were carried out in order to prove the lack of cross-activation of PKA when using the EPAC activator.

In the second part of this work, we attempted to identify intracellular signals that connect PKA and/or EPAC activation with NHE3 inhibition. Using different approaches, the effects of PKA and EPAC activation on phospholipase C (PLC), protein kinase B (PKB/Akt) and extracellular signal-regulated kinases 1/2 (ERK1/2) were analyzed.

5. Material and Methods

5. 1 Preparation of mouse kidney slices

Male NMRI mice, about 8 weeks old, were fed a normal diet (Kliba, NAFAG, Switzerland) and had free access to normal tap water. Mice were anaesthetized by intraperitoneal administration of 80 mg/kg body weight ketamine (Narketan 10, Chassot, Belp, Switzerland) and 33 mg/kg body weight xylazine (Rompun, Bayer, Leverkusen, Germany). To remove blood from kidneys, animals were perfused through the left ventricle with 50 ml of warm (30°C) sucrose/phosphate buffer (140 mM sucrose, 140 mM NaH_2PO_4/NaH_2PO_4, pH 7.4). Kidneys were harvested and the adhering connective tissue and extrarenal tissues were removed. Then, coronal slices of about 1 mm in thickness were prepared. From each kidney six to seven slices were prepared. Slices were transferred into 4 ml of pre-warmed Hank's buffer (110 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 4 mM Na-acetate, 1 mM Na-citrate, 6 mM glucose, 6 mM L-alanine, 1 mM NaH_2PO_4, 3 mM Na_2HPO_4, 25 mM $NaHCO_3$, pH 7.4, gassed with 5% CO_2 and 95% O_2) and allowed to adapt for 10 min at 37°C in a water bath prior incubation with the indicated agonists. During the whole course of the experiment all solutions were gassed with 5% CO_2/95% O_2 and the pH was kept constant at pH 7.4 ± 0.1. We have previously shown that under these conditions, the proximal tubules retain their morphological characteristics up to 2 hours incubation; in addition, the expression of several cotransporters remained unchanged in this time window (Bacic, Schulz et al. 2003).

5. 2 Treatment of kidney slices

Slices were incubated for 30 min with 200 µM ATP in the absence or presence of 100 µM 8-Br-cAMP, and the indicated concentrations of the PKA (6-MB-cAMP) and/or the EPAC (8-pCPT-2`-O-Me-cAMP) activating cAMP analogs (Fig. 18). Where indicated, slices were preincubated for 10 min with 50 µM of the PKA inhibitor H89 or 20 µM of the MEK1/2 inhibitor PD098059, prior to addition of the cAMP analogs.

After treatment, slices were processed either for immunohistochemistry or for preparation of total homogenates and/or brush border membrane vesicles (BBMV). Homogenates were tested for PKA activity, whereas BBMV were processed for Western blots and determination of Na^+/H^+ exchanger activity.

cAMP analogs were obtained from BIOLOG Life Science Institute (Germany) and were resuspended in water. All other chemicals were purchased from SIGMA and were resuspended in water with the only exception of PD098059 which was prepared in DMSO.

All experiments were performed at least three times with two kidneys from two different animals.

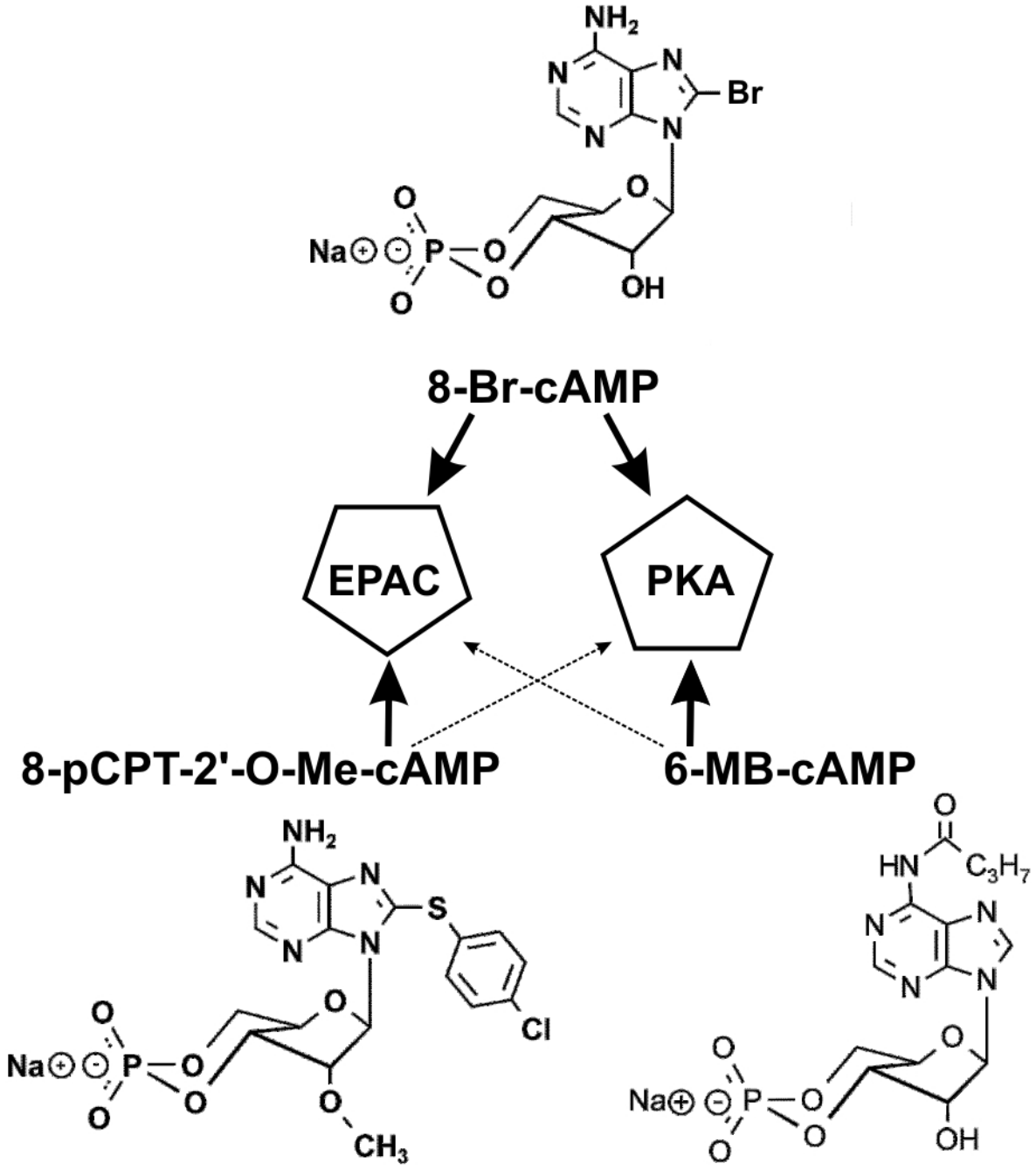

Figure 18: Chemical structures of the cAMP analogs

8-Br-cAMP activates both PKA and EPAC. 8-pCPT-2'-O-Me-cAMP preferentially activates EPAC, while 6-Monobutyryl-cAMP activates PKA.

5. 3 Immunohistochemistry in kidney slices

At the end of the incubation time, kidney slices were transferred to a fixation solution and processed for immunostaining as described previously (Dawson, Gandhi et al. 1989; Bacic, Schulz et al. 2003). The fixative solution consisted of 3% paraformaldehyde and 0.05% picric acid in a 6:4 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol/l with sucrose) and 10% hydroxyethyl starch in saline (HAES steril; Fresenius, Bad Homburg, Germany). Slices were fixed for 4 h on ice. After fixation, slices were rinsed a few times with phosphate-buffered saline (PBS), mounted onto thin cork plates and immediately frozen in liquid propane, cooled with liquid nitrogen. Cryosections mounted on chromalum/gelatin-coated glass slides were processed for immunofluorescence as previously reported (Bacic, Schulz et al. 2003). Cryosections were incubated with antibodies against NHE3 (1:500; antisera # 1568 kindly provided by Dr. O Moe; University of Texas Southwestern, Dallas, Texas), NaPi-IIa (1:500; (Custer, Lotscher et al. 1994) or an affinity-purified antibody against a C-terminal peptide of EPAC1 (1:20; Santa Cruz Biotechnology) as previously reported. To control for the specificity of the anti-EPAC immunostaining, the antiserum was incubated over night at 4°C in the absence/presence of the synthetic antigenic peptide (10 mg/ml) before application. Double staining with β-actin was performed by adding rhodamine-phalloidin (1:50; Molecular Probes) together with the secondary antibodies.

The immunostaining protocol was as follows: sections were first preincubated for 10 min in a blocking solution that contained 3% defatted milk powder and 0.02% Triton X-100 in PBS. After rinsing with PBS, sections were incubated overnight at 4°C with the indicated antibody diluted in blocking solution. Samples where then rinsed three times with PBS and covered for 45 min at room temperature in the dark with swine anti-rabbit IgG conjugated to FITC (Dakopatts, Glostrup, Denmark) or goat anti-mouse IgG conjugated to FITC (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) diluted 1:50 in PBS/milk powder. After several washes with PBS, the sections were finally plated on coverslips using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo-2.2.2-octane (DABCO; SIGMA) as a fading retardant, and studied on an epifluorescence microscope (Polyvar, Reichert-Jung).

5. 4 Preparation of total homogenates and brush border membrane vesicles (BBMV)

Kidney slices were homogenized and BBMV prepared using the Mg^{2+}-precipitation technique (Biber, Stieger et al. 1981). In brief, at the end of the incubation period slices were transferred into 800 µl ice-cold homogenization buffer containing 300 mM mannitol, 5 mM EGTA and 12 mM Tris-HCl, pH 7.1. Slices were homogenized on ice using a polytron (Kinematica). Upon addition of 1120 µl water and 23

μl of 1M $MgCl_2$ (12 mM final) samples were precipitated on ice for 20 min. Then, samples were centrifuged in a Sorvall centrifuge (rotor SS34) for 15 min at 4500 rpm. Small aliquots of supernatants, representing total homogenates, were immediately frozen, whereas the rest were further centrifuged at 18500 rpm for 30 min. Pellets were resuspended in 1 ml of membrane buffer (300 mM mannitol, 20 mM Hepes-Tris, pH 5.5 and 2 mM $MgCl_2$) and centrifuged again at 18500 rpm for 30 min. After removing carefully the supernatants, pellets containing BBMV were diluted in 300 μl of membrane buffer. Finally, protein concentration was measured with a Biorad protein determination kit.

5. 5 Determination of NHE activity in BBMV

Kidney slices were homogenized and BBMV prepared as indicated above. BBMV were diluted in a buffer containing 280 mM mannitol, 5 mM MES, 2 mM $MgCl_2$ and adjusted to pH 5.5 with N-methyl-D-glucamine. Acridine-orange quenching was measured to monitor changes in transmembrane ΔpH as described previously (Cassano, Stieger et al. 1984). Measurements were performed in a Shimadzu RF-5000 spectrofluometer equipped with a thermostatized cuvette (kept at 25°C), a stirring system and a vertical inlet that allows a direct injection of test solutions into the cuvette without interruption of the fluorescence measurement. Acridine-orange was excited at 493 nm and emission was monitored at 530 nm. The cuvette was filled with 2 ml buffer (240 mM mannitol, 20 mM HEPES and 2 mM $MgCl_2$ adjusted to pH 7.5 with N-methyl-D-glucamine), containing a final concentration of 6 μM acridine-orange. The experiment was started by injecting 30 μl of BBMV suspension containing 150 μg protein. After 60 sec of equilibration, Na^+/H^+ exchange activity was initiated by injection of 80 μl of 2 M Na^+-gluconate resulting in a steep increase in quenching. NHE activity was calculated as ratio of ΔpH/min over Q, where Q is the initial quenching after injection of BBMV. All experiments were done at least in quadruplicates and repeated four times with 2 animals per experiment.

5. 6 Western blot of BBMV

Twenty μg of BBMV were solubilized (1:1) in Laemmli sample buffer (0.38 M Tris, 400 mM DTT, 4 mM EDTA, 8% SDS, 40% glycerol). Proteins were separated into a 9% polyacrylamide gel in the presence of 24.7 mM Tris, 200 mM Glycine and 1% SDS. Then, proteins were transferred electrophoretically from the gels to nitrocellulose membranes (Protran BA 83 Cellulosenitrate; Schleicher and Schuell) in the presence of 24.7 mM Tris, 192 mM Glycine and 20% methanol. Nitrocellulose membranes were preincubated for 2 h at room temperature in blocking solution containing 5% milk powder and 1% Triton X-100 in TBS (150 mM NaCl and 25 mM Tris) pH 7.4. Then, blots were incubated overnight at 4°C with the following primary antibodies: polyclonal anti-NaPi-IIa generated in our laboratory (Custer, Lotscher et al. 1994) diluted 1: 6000, polyclonal anti-NHE3 (kindly provided by Dr. O Moe; University of Texas

Southwestern, Dallas, Texas) diluted 1:1000, polyclonal serine/threonine PKA substrate antibody (Cell Signaling) diluted 1:1000 and polyclonal PKB substrate antibody (Cell Signaling) diluted 1:1000; all antibodies were diluted in blocking solution. After washing in TBS and blocking for 30 min at room temperature, blots were incubated for 2 h at room temperature with a secondary donkey anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Life Sciences); the secondary antibody was diluted 1:12000 in blocking solution. Antibody binding was detected with the peroxidase/luminal enhancer kit (Pierce, Rockford, USA) before exposure to X-ray film (Kodak).

5. 7 Determination of PKA activity in cortex homogenates

PKA activity in total homogenates was determined using the SignaTECT PKA assay system (Promega). This system uses a biotinylated peptide derived from pyruvate kinase (LRRASLG) as PKA substrate. Homogenate samples (20 μg) were incubated with 40 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 0.1 mg/ml BSA, 100 μM biotinylated PKA substrate, 100 μM ATP and 10 μCi/μl γ^{32}P-ATP (Hartmann Analytic), in a final volume of 25 μl. Reactions were carried out for 5 min at 30°C. The kinase reaction was terminated by addition of 12.5 μl of the termination buffer (7.5 M guanidine hydrochloride). Then, the ^{32}P-labeled substrate was purified from the reaction mix by spotting 10 μl of each kinase reaction onto the Biotin Capture Membranes provided by the kit. Membranes were sequentially washed in 2 M NaCl (4x, 2 min each), 2 M NaCl in 1% H_3PO_4 (4x, 2 min each) and deionized water (2x, 2 min each). Membranes were air-dried and the incorporation of ^{32}P was measured in a scintillation counter.

5. 8 Determination of PLC activity in BBM

PLC activity in BBM was determined using the Amplex Red phosphatidylcholine-specific phospholipase C assay kit (Molecular Probes). In this enzyme-coupled assay, PC-PLC activity is monitored indirectly using 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent), a sensitve fluorogenic probe for H_2O_2. First, PC-PLC converts the phosphatidylcholine (lecithin) substrate to form phosphocholine and diacylglycerol. After the action of alkaline phosphatase, which hydrolizes phosphocholine, choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidise, reacts with Amplex Red reagent to generate the highly fluorescent product, resorufin. Resorufin has absorption and fluorescence emission maxima of 563 nm and 587 nm, respectively. 5 μg of BBM was used per sample, the assay was done in triplicates.

5. 9 Cell culture: OK cells

Opossum kidney cells (OK cells, clone 3B2, passage 10-35) were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum, 22 mM $NaHCO_3$, 20 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C. All cell culture supplies were obtained from GIBCO Invitrogen Corp. Cells were trypsinized every week and fed every 2 to 3 days until reaching confluency.

5. 10 Determination of NHE activity in OK cells: intracellular pH measurements

OK cells were grown to subconfluency on glass slides placed in 10 cm-dishes. Individual slides were transferred to a heated perfusion chamber maintained at 37°C on an inverted microscope (Zeiss Axiovert 200) and attached to a free-flow perfusion system that allowed rapid changes of superfusate. All solutions were kept at 37°C using a feedback heating system. After mounting, cells were incubated for 15 min at 37°C with a standard HEPES solution (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 2 mM KH_2PO_4, 5 mM Glucose, 32.2 mM HEPES, pH 7.4) containing 10 µM of the pH-sensitive dye BCECF-AM (Molecular Probes, Portland, Oregon) and then washed with HEPES to remove any de-esterified dye. After washing, areas of interest were identified at 40x magnification using the high speed ratio program and these areas along with the images were recorded to a hard disk. Cells were excited with 490 and 440 nm light while monitoring the emission at 535 nm. The ratiometric emission of 490/440 was converted to pH_i after calibration (Thomas, Buchsbaum et al. 1979) using the high K^+/nigericin technique (Roos and Boron 1981; Winter, Schulz et al. 2004). In brief, following a 20 mM NH_4Cl prepulse, cells were washed in the absence of Na^+ (HEPES buffer with NaCl replaced by 105 mM of *N*-methyl-D-glucamine); then the Na^+/H^+ exchanger activity was calculated from the steep phase of intracellular alkalinization upon readdition of Na^+. To allow for direct comparison, ΔpH/min was calculated only for intracellular pH values in the range between pH 6.50 and 6.80. All experiments were performed as paired experiments with measurement of Na^+/H^+ exchange activity before and after a 15 min period of incubation of cells with 50 µM 8-Br-cAMP, EPAC and/or PKA activators. Control cells were incubated only with standard HEPES solution. Data are presented as mean ± SEM.

5. 11 Determination of NaPi-IIa activity in OK cells: isotope flux (^{32}P-uptakes)

Confluent OK cells plated on 12-multiwell plates were incubated for 4 h with either 10 nM PTH (bovine synthetic fragment 1-34, SIGMA), or the indicated concentrations of cAMP analogs. Uptakes were performed by incubating the cells in the presence of 0.25-0.5 µCi $^{32}PO_4$/ml (Hartmann Analytic) as already described in detail (Sorribas, Markovich et al. 1995). In brief, cells were washed 2x with 1 ml of

pre-warmed wash solution containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $Ca_2Cl.2H_2O$, 1.2 mM $MgSO_4$, 10 mM HEPES, pH 7.4. Reactions were started by addition of 500 µl uptake solution/well; this uptake solution consisted of wash solution containing 100 µM PO_4HK_2:PO_4H_2K and 0.25-05 µCi/ml ^{32}P. Upon incubation for 10 min at room temperature, reactions were stopped by washing the cells 4 times with ice-cold stop solution (137 mM NaCl, 14 mM Tris-HCl, pH 7.4). Cells were then solubilized with 1 ml of 0.05% Na-deoxycholate in 0.01 M NaOH. After neutralization with 200 µl of 1 M HCl, cells were scraped off and homogenized by pipetting, before transferring 200 µl to a scintillation vial to count ^{32}P-incorporation. Transport rates are expressed as % of the control (untreated) samples.

5. 12 OK cell lysate preparation and Western blot

Confluent cultures were incubated with cAMP analogs (50 µM) for the indicated times or with 1-34 PTH (10 nM) for 4 h. Cells were lysed in TBS (150 mM NaCl, 25 mM Tris, pH 7.4) containing Igepal (1:200) and protease inhibitor cocktail (1:100). Lysates were centrifuged at 5000 rpm at 4°C for 5 min to eliminate nuclei and cellular debris. The supernatants were carefully removed and their protein concentration determined with a Biorad protein determination kit. About 50 µg lysates were subjected to Western blot as indicated above. Nitrocellulose membranes (Schleicher & Schuell) were incubated overnight at 4°C with a polyclonal antibody against the Opossum NHE3 (1:1000; kindly provided by Dr. O Moe; University of Texas Southwestern, Dallas, Texas) as well as with a polyclonal antibody against the Opossum NaPi-IIa (1:6000; kindly provided by Dr. E. Lederer; Division of Nephrology, University of Louisville, Louisville, Kentucky). Upon incubation with HRP-linked secondary antibodies, immunoreactive signals were detected by chemiluminiscence (ECL Amersham Pharmacia Biotech).

5. 13 Surface biotinylation

Confluent OK cell cultures grown in 10-cm plates were incubated for 15 min or 4 h with cAMP analogs (50 µM) as well as with 1-34 PTH (10 nM). Cells were washed 4 times with PBS (containing 0.88 µM Ca^{2+} and 0.5 µM Mg^{2+}). Then, surface-expressed proteins were biotinylated for 1 h with NHS-SS-Biotin (1.5 mg/ml, PIERCE) as reported (Di Sole, Cerull et al. 2004). After incubation with 100 mM Glycin, cells were lysed in RIPA-buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 5 µg/ml Leupeptin, 5 µg/ml Pepstatin A). Lysates were centrifuged at 2500 rpm at 4°C for 25 min, and the supernatants incubated overnight with 50 µl of pre-washed Streptavidin-beads (PIERCE). After centrifugation at 10000 rpm for 20 sec the pellets were washed sequentially with buffer A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA), buffer B (50 mM Tris-HCl, pH 7.4, 500 mM NaCl) and buffer C (50 mM Tris-HCl, pH 7.4). Proteins were eluted by addition of 30 µl of 2x loading buffer (containing 60 µg/µl DTT) and incubation at 95°C for 3 min. After centrifugation at 3000

rpm for 10 min, supernatants were loaded on SDS-PAGE and Western blot was performed with NHE3 antibodies as described above.

5. 14 NHE3 phosphorylation

OK cells were plated in 10-cm plates. After reaching confluency, cells were phosphorylated following published protocols (Di Sole, Cerull et al. 2004). Thus, cells were serum-starved overnight followed by incubation for 1 h in phosphate-free medium (1.8 mM $CaCl_2$, 5.36 mM KCl, 109.5 mM NaCl, 0.8 mM $MgSO_4$, 44 mM $NaHCO_3$, 25 mM D-glucose, 4 mM glutamine, plus 4x amino acids for basal MEM, 1x non-essential amino acids and 4x vitamins for basal MEM). Then, cells were incubated for 2 h in the presence of 100 μCi $^{32}PO_4$/ml. After treatment for 30 min with 50 μM 8-Br-cAMP as well as with 50 μM of the PKA and EPAC activators, cells were lysed in 300 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA and 0.5 mM DTT, plus 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. Lysates were centrifuged for 30 min at 40000 rpm, and the supernatants were diluted 1:1 in water to reduce salt concentration. Then, supernantants were subjected to immunoprecipitation using the polyclonal antibody raised against the Opossum NHE3. Briefly, lysates were first incubated for 4 h at 4°C with the NHE3 antibody (1:200). Then, 40 μl of protein G/A agarose beads (Oncogene) were added and samples were incubated overnight at 4°C. After 6 washes with lysis buffer, bound proteins were finally eluted in 35 μl of 1x loading buffer (containing 1% β-mercaptoethanol) by incubating at 85°C for 10 min. Samples (25 μl) were run in a 9% SDS-PAGE gel and transferred to nitrocellulose membranes. Incorporation of ^{32}P was detected by exposition of an X-ray film at -80°C.

6. Results

6. 1 Localization of EPAC1 in the BBM of proximal tubules

The localization of EPAC1, NaPi-IIa and NHE3 was analyzed in mouse kidney sections (Fig. 19). The left panel of Fig. 19A shows the immunostaining using an affinity-purified antibody against a C-terminal peptide of EPAC1. EPAC1 was detected in S1, S2 and S3 segments of proximal tubules (PT), where the signal was concentrated in the BBM (white signal). Within the PTs, the strongest staining was found in S2 segments and the weakest in S3. No signal was detected in glomeruli or distal parts of the nephron (data not shown). The right panel of Fig. 19A shows the immunosignal that was obtained when the antibody was preincubated with the antigenic peptide prior to incubation with the tissue. Preincubation with the peptide blocked the fluorescent signal.

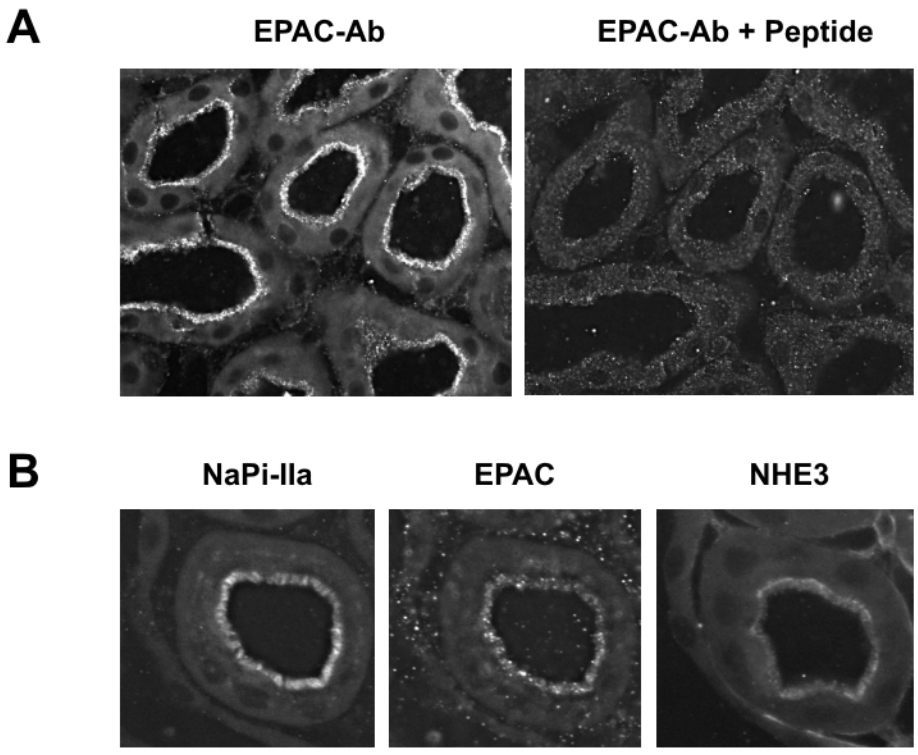

Figure 19: EPAC1 localization in mouse kidney

A) EPAC1 staining was detected in proximal tubules (left panel). The signal was attenuated after preincubation of antibody with the antigenic peptide (right panel). **B)** Consecutive cryosections were stained for NaPi-IIa, EPAC and NHE3. All three proteins were localized in the BBM of proximal tubular cells.

In order to compare the localization of NaPi-IIa, NHE3 and EPAC1, consecutive kidney sections were stained with the corresponding antibodies (Fig. 19B). NaPi-IIa, EPAC and NHE3 signals were all located in the BBM (white signal).

6. 2 Effect of cAMP analogs on the activity and expression of NHE3 in OK cells

The activity of the endogenous NHE3 in OK cells was examined by determining the Na^+-dependent pH recovery rate using the pH-sensitive BCECF dye (Fig. 20). Fig. 20A shows a scheme of the standard protocol as well as typical pH traces of 20 individual cells. The experiment consisted of two parts. In the first part, the pH recovery rate after the NH_4Cl-induced acidification was measured in the absence of agonists and 15 min after addition of BCECF (indicated by arrow). These control values were taken as 100% for every individual experiment. In the second part, the pH recovery rate was measured after 15 min incubation with either 8-Br-cAMP (shown here as an example) or EPAC and PKA activators (see Fig. 18). As indicated, activators were applied together with a new load of BCECF. Fig. 20B shows the averaged values of the pH recovery rates, expressed as percentage of the control. The pH recovery rate, and therefore the activity of NHE3, was reduced by about 50% after incubation of the cells with 8-Br-cAMP as well as with the EPAC and PKA activators.

The effect of the different cAMP analogs on the total amount of NHE3 was analyzed by Western blot using a NHE3 antibody. At least three independent experiments were performed, each of which was carried out in duplicate. As shown in Fig. 20C, similar amounts of NHE3 were detected in lysates obtained from control cells, or from cells treated for 15 min with either 8-Br-cAMP or the EPAC and PKA activators.

The effect of the different cAMP analogs on the amount of membrane-bound NHE3 was also studied. For that purpose, OK cells were incubated for either 15 min or 4 h in the absence or presence of either 8-Br-cAMP or the EPAC and PKA activators; in addition, cells were incubated for 4 h with 1-34 PTH. Upon purification of surface-expressed proteins by biotin-streptavidin precipitation, the expression of NHE3 in the plasma membrane was analyzed by Western blot using the NHE3 antibody mentioned above. At least three independent experiments were performed. As shown in Fig. 20D, incubation with PTH led to a reduction of the amount of membrane-bound NHE3. However, incubation with the different cAMP analogs, for either 15 min or 4 h, did not result in changes of surface-expressed NHE3.

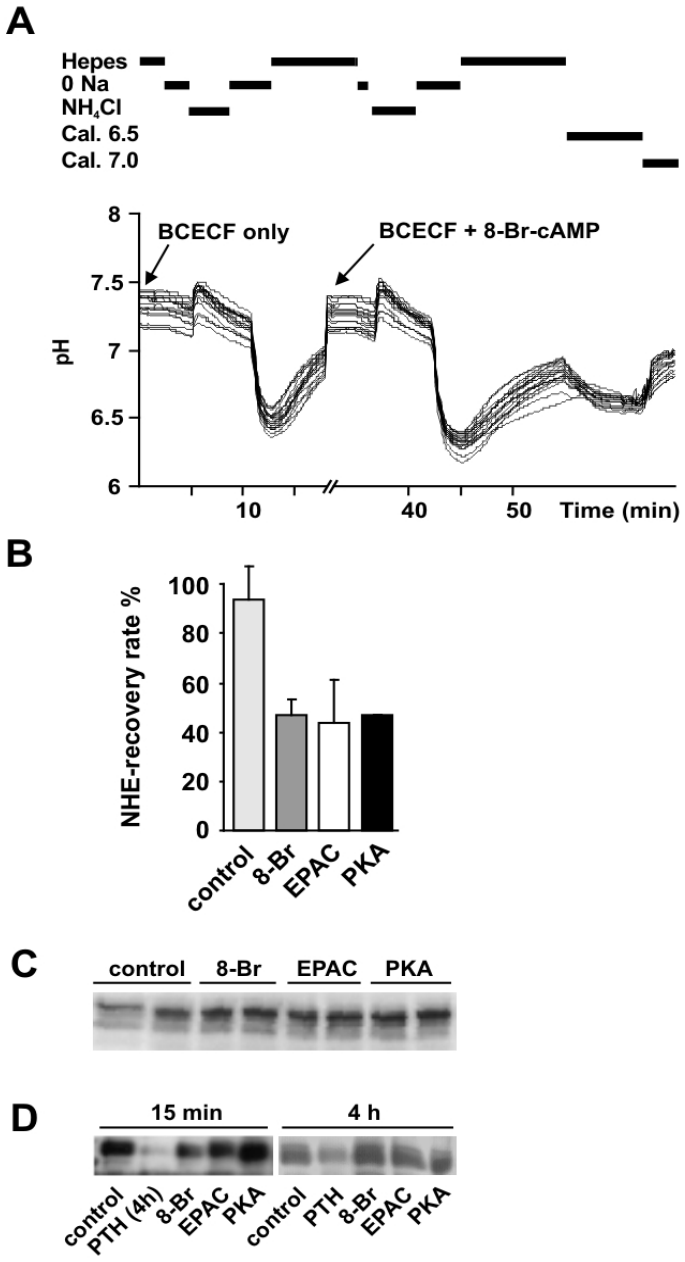

Figure 20: NHE3 activity and expression in OK cells

A) Example of a typical intracellular pH trace: cells were preincubated with BCECF, followed by 15 min incubation in the absence or presence of 50 μM 8-Br-cAMP; about 20 OK cells were monitored per experiment. **B)** Effect of the different cAMP analogs on the Na^+-dependent intracellular pH recovery (NHE activity). Data are presented as mean ± SEM (n= 4). **C)** Total expression of NHE3: cells were incubated in duplicate in the absence or presence of 50 μM 8-Br-cAMP, EPAC or PKA activator for 15 min. Cell lysates were processed for Western blot with an anti-NHE3 antibody (n= 3). **D)** Surface expression of NHE3: cells were incubated for 15 min or 4 h in the absence or presence of 50 μM 8-Br-cAMP, EPAC or PKA activators as well as with 10 nM PTH; PTH was always applied for 4 h. Upon biotinylation and streptavidine precipitation, samples were subjected to SDS-PAGE and incubated with an anti-NHE3 antibody, (n= 4 and 3, respectively).

6. 3 Effect of cAMP analogs on NHE activity in BBMV (kidney slices)

The NHE activity in BBMV isolated from slices of mouse kidney cortex was measured by acridine-orange fluorimetry (Fig. 21). All experiments were done at least in quadruplicates and repeated four times with 2 animals per experiment. Fig. 21A shows a superposition of original acridine-orange traces obtained from BBMV isolated from slices incubated in the absence (dashed line) or presence (full line) of the PKA activator. Injection of Na^+-gluconate to a cuvette containing BBMV and acridine-orange resulted in an increase in quenching. NHE activity was calculated as ratio of ΔpH/min over Q, where Q is the initial quenching after injection of BBMV. Incubation with the PKA activator resulted in a reduction of the NHE activity, as indicated by a shallower slope compared to control. As shown in Fig. 21B, both the PKA (black bars) and EPAC (white bars) activators induced a dose-dependent inhibition of NHE. At the smallest concentration tested (10 μM), both agonists led to a small but detectable inhibition of NHE (about 5 to 10%), whereas at the higher concentration tested (100 μM) the PKA and EPAC activators reduced the NHE activity by about 30 to 40%. Both activators led to very similar percentage of inhibition.

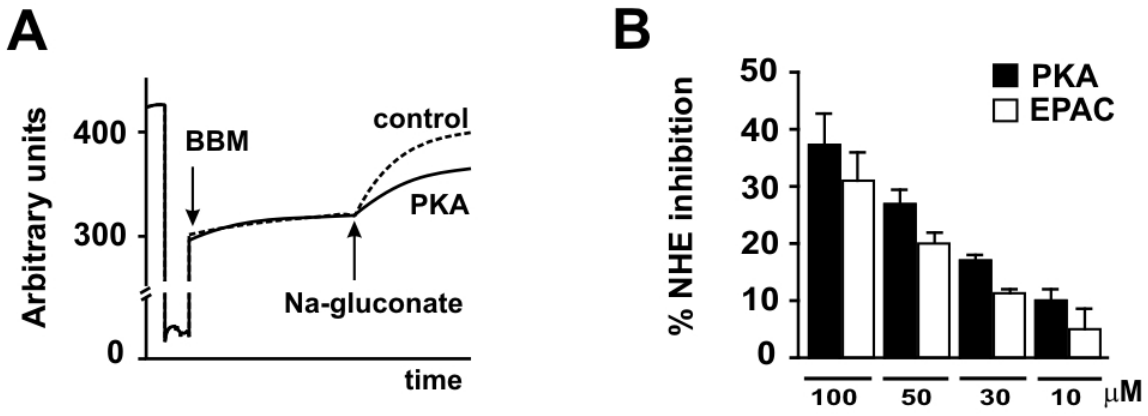

Figure 21: NHE activity in mouse BBM

Kidney slices were incubated for 30 min with the indicated concentrations of EPAC and PKA activators. BBMV were then prepared and the NHE activity was measured as described in Material and Methods. **A)** Reproduction showing a superposition of original acridine-orange fluorescence traces obtained with BBMV isolated from kidney slices incubated in the absence or presence of PKA activator. **B)** Concentration-response of both activators over a range of 10 to 100 μM. Data are presented as mean ± SEM, (n= 4).

6. 4 Specificity of the effects of the cAMP analogs

The specificity of the cAMP analogs regarding their ability to activate PKA in kidney slices was analyzed using a PKA specific assay (Fig. 22A). Kidney slices were first treated in the absence (-) or presence (+) of 50 μM of the PKA (black bars) or EPAC (white bars) activators. Slices were then homogenized, and the homogenates tested for their ability to induce phosphorylation, in the presence of γ^{32}P-ATP, of a biotinylated PKA substrate. Upon purification with streptavidin, the incorporation of γ^{32}P-ATP in the PKA substrate was counted. Data are presented as pmol of incorporated ATP per min and μg of protein. As shown in Fig. 22A, homogenates derived from slices incubated in the absence of cAMP analogs (-) were able to induce phosphorylation of the exogenous PKA substrate. Quantification of 3 independent experiments indicated that this basal PKA activity was about 0.130 pmol ATP/min/μg protein. The ability to phosphorylate the exogenous PKA substrate was twice as much in the homogenates derived from samples incubated with the PKA activator as that of the control samples. However, the homogenates from slices incubated with the EPAC activator did not induce phosphorylation over basal levels. Preincubation with the PKA inhibitor H89 reduced the basal PKA activity in homogenates and blunted the phosphorylation promoted by the PKA activator (data not shown).

The ability of the cAMP analogs to activate PKA in kidney slices was also analyzed indirectly, by testing the presence of phosphorylated PKA substrates using a PKA substrate antibody. As shown in Fig. 22B, this antibody identified two phosphorylated PKA substrates of around 60 and 240 kDa in BBM derived from control (untreated) samples. Incubation with the PKA activator led to hyperphosphorylation of the 240 kDa-substrate and to the appearance of three more phosphorylated bands (indicated by arrows): two bands in the area between the 100 and 250 kDa markers and one between the 75 and 100 kDa markers. However, incubation with the EPAC activator did not induce the appearance of new phosphorylated substrates as compared with control samples.

The specificity of the PKA and EPAC effects was also studied by testing the effect of the PKA inhibitor H89 on both responses (Fig. 22C). The PKA- (black bars) and EPAC- (white bars) dependent inhibition of NHE activity was analyzed in the absence (-) or presence (+) of 50 μM H89. The NHE activity was inhibited by about 34% after treatment with the PKA activator, and this inhibition was abolished in the presence of H89. On the other hand, NHE activity was inhibited by about 22% after treatment with the EPAC activator alone, and such inhibition was similar in the absence or presence of H89.

The PKA assay and the effect of H89 on the NHE inhibition induced by the PKA and EPAC activators were also performed upon incubation of kidney slices with 100 μM (instead of 50 μM) of each cAMP analog. As expected, the ability of homogenates to phosphorylate the PKA substrate was stronger in samples incubated with 100 μM of the PKA activator than in control samples (Fig. 22D). Moreover, homogenates derived from slices incubated with 100 μM of the EPAC activator were also able to induce phosphorylation of the exogenous PKA substrate above basal levels, suggesting that at this concentration the EPAC activator cross-activates PKA. Similar to the PKA effect, the NHE inhibition

induced by 100 μM of the EPAC activator was prevented by preincubation with the PKA inhibitor H89 (Fig. 22E).

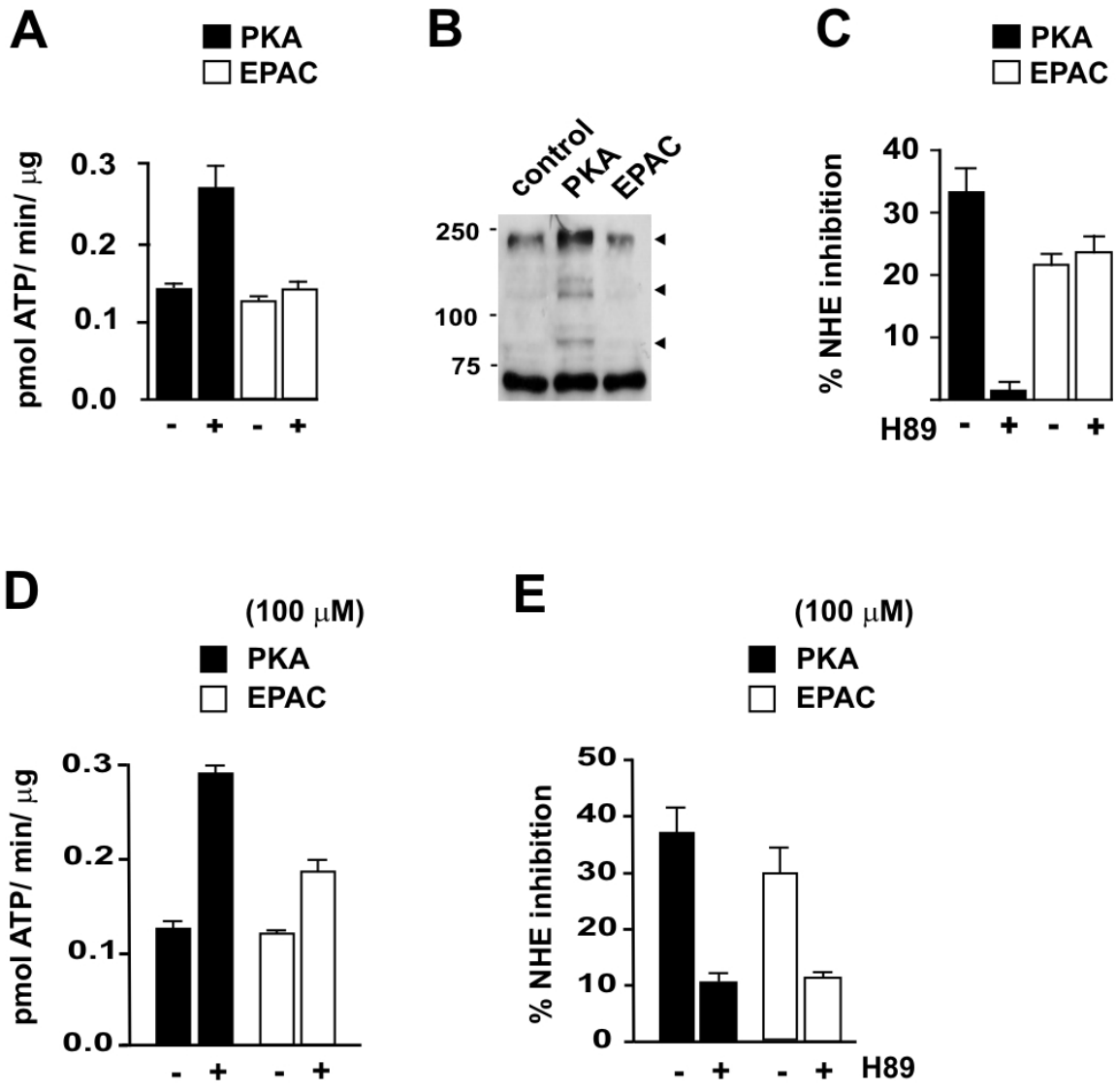

Figure 22: Specificity of the effects of cAMP analogs

A) Determination of PKA activity in homogenates isolated from kidney cortex slices incubated in the absence or presence of 50 μM of the PKA and EPAC activators (n= 3). **B)** Western blot with an anti-PKA-substrate antibody of BBMV incubated in the absence or presence of 50 μM PKA or EPAC activator. Substrates phosphorylated upon PKA treatment are indicated by arrows. **C)** Effect of H89 on the PKA- and EPAC-dependent NHE inhibition: kidney slices were incubated in the absence or presence of 50 μM H89 for 5 min, prior to addition of 50 μM of the PKA or EPAC activators (n= 4). **D)** Determination of PKA activity in homogenates isolated from kidney cortex slices incubated in the absence or presence of 100 μM of cAMP analogs (n= 3). **E)** Effect of H89 on the PKA- and EPAC-dependent NHE inhibition: kidney slices were incubated in the absence or presence of 50 μM H89 for 5 min, prior to addition of 100 μM of cAMP analogs (n= 3).

6. 5 PKA and EPAC effects are not additive

In order to test whether the PKA and the EPAC pathways have an additive effect, kidney slices were incubated with the PKA and EPAC activators either alone or in combination, and their effect on NHE activity was determined (Fig. 23). Simultaneous activation of both pathways did not induce a stronger inhibition of NHE activity than the independent activation of either pathway. This could suggest that PKA and EPAC compete for common downstream effectors.

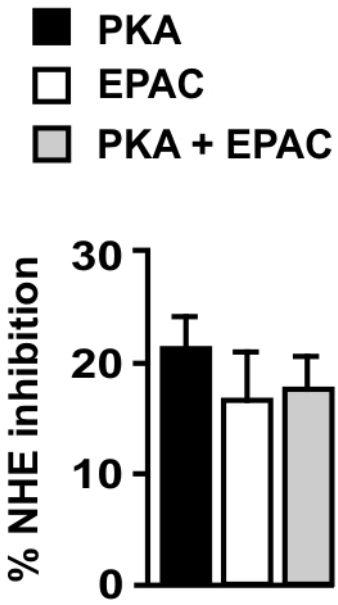

Figure 23: Simultaneous PKA and EPAC activation
Effect of incubation with 50 μM PKA and/or EPAC activator.

6. 6 Searching for downstream effectors of PKA and EPAC

We first studied whether PKA and/or EPAC activate the phospholipase C (PLC) pathway. The PLC activity of BBM isolated from slices treated with different analogs was measured indirectly using a sensitive fluorogenic probe to generate a highly fluorescent product which has absorption and fluorescence emission maxima of 563 and 587 nm, respectively. Activity is represented as percentage of control. As shown in Fig. 24A, BBM isolated from samples treated with the PKA activator did not induce PLC activity over basal levels. On the other hand, BBM purified upon incubation of kidney

samples with the EPAC activator induced a moderate but statistically non-significant (p= 0.13) increase of PLC activity.

To study the potential role of PKB in the PKA- and EPAC-dependent response, we analyzed kidney cortex homogenates for changes in the pattern of expression of phosphorylated PKB substrates. For that purpose, Western blots of homogenates using a phospho-(serine/threonine)-PKB substrate antibody were performed (Fig. 24B). This antibody detected several phosphorylated PKB substrates in homogenates isolated from untreated kidney slices. The two most consistently detected substrates had an apparent molecular weight of around 45 and 100 kDa. Quantification of 4 (PKA) or 5 (EPAC) independent experiments indicated no significant changes in the phosphorylation pattern upon incubation of kidney slices with the different cAMP analogs (Fig. 24C).

To study the potential implication of the ERK1/2 kinase pathway in the NHE inhibition generated by the cAMP analogs, the effect of the MEK1/2 inhibitor PD098059 was investigated (Fig. 24D). The effect of both the PKA (black bars) and EPAC (white bars) activators on NHE activity were compared in the absence (-) or presence (+) of 20 μM PD098059. As shown in Fig. 24D, both analogs reduced the NHE activity by about 30%. Incubation with PD098059 partially prevented the PKA effect (12% inhibition), whereas it fully blocked the inhibition generated by the EPAC activator. We could not observe changes in the phosphorylation state of ERK1/2 upon activation of either pathway (data not shown).

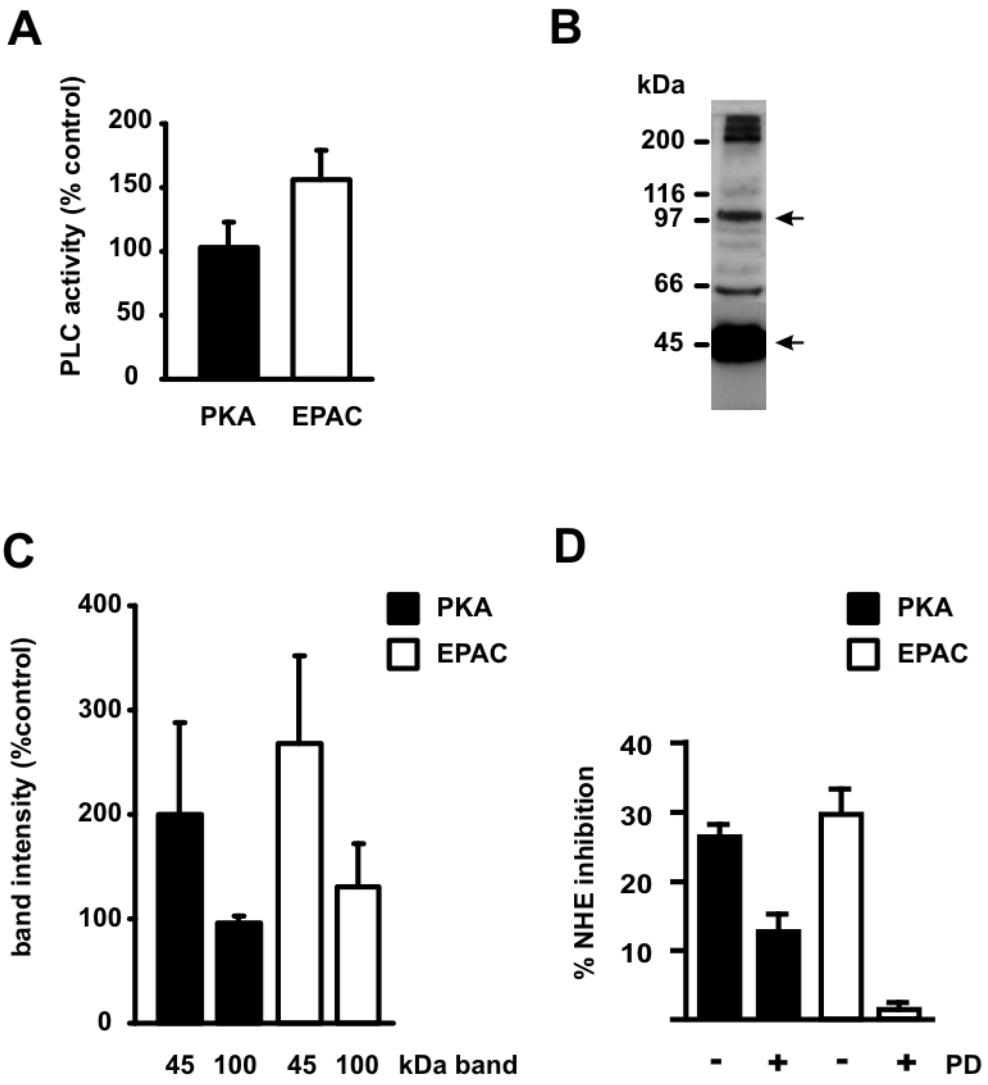

Figure 24: Downstream effectors of PKA and EPAC

A) Measurements of PLC activity using the Amplex Red phosphatidylcholine specific PLC assay kit. BBM were treated with PKA (n=4) or EPAC activator (n=5). Bars express the ratio of treated samples/controls in %. **B)** Example of phosphorylated PKB substrates (bands of 42 and 100 kDa) detected by Western blot of untreated homogenates. **C)** PKB substrates: quantification of several experiments with the effect of the PKA (n=4) or EPAC activator (n=5), was determined. Data is expressed in % of control values. **D)** Effect of PD098059 on the PKA- and EPAC-dependent NHE inhibition: kidney slices were incubated in the absence or presence of 20 μM PD098059 for 5 min, prior to addition of 50 μM of the PKA or EPAC activators (n= 3).

6. 7 Effect of cAMP analogs on the pattern of NHE3 expression

To study whether the PKA- and EPAC-induced inhibition of NHE activity in kidney was due to changes in the amount of the exchanger, BBM isolated from kidney slices were analyzed by Western blot with anti-NHE3 antibody (Fig. 25A). Four independent experiments were performed, in which kidney slices were incubated for 30 min in the absence or presence of 50 μM of the PKA or EPAC activator. Samples

were processed together for Western blot. The amount of NHE3 was similar in control and treated samples.

The pattern of expression of NHE3 upon incubation of kidney slices with the cAMP analogs was analyzed by immunohistochemistry (Fig. 25B). For that purpose, kidney slices were incubated with the same anti-NHE3 antibody used in Western blots described above as well as with phalloidin. The NHE3 signal is shown in green and the actin signal in red. In control samples, NHE3 was concentrated in the BBM where it was co-expressed with actin, as indicated by the yellow color of the merged picture. A similar pattern of expression was detected in kidney slices incubated with the PKA or the EPAC activators, suggesting that NHE3 remained expressed in the BBM in all experimental conditions.

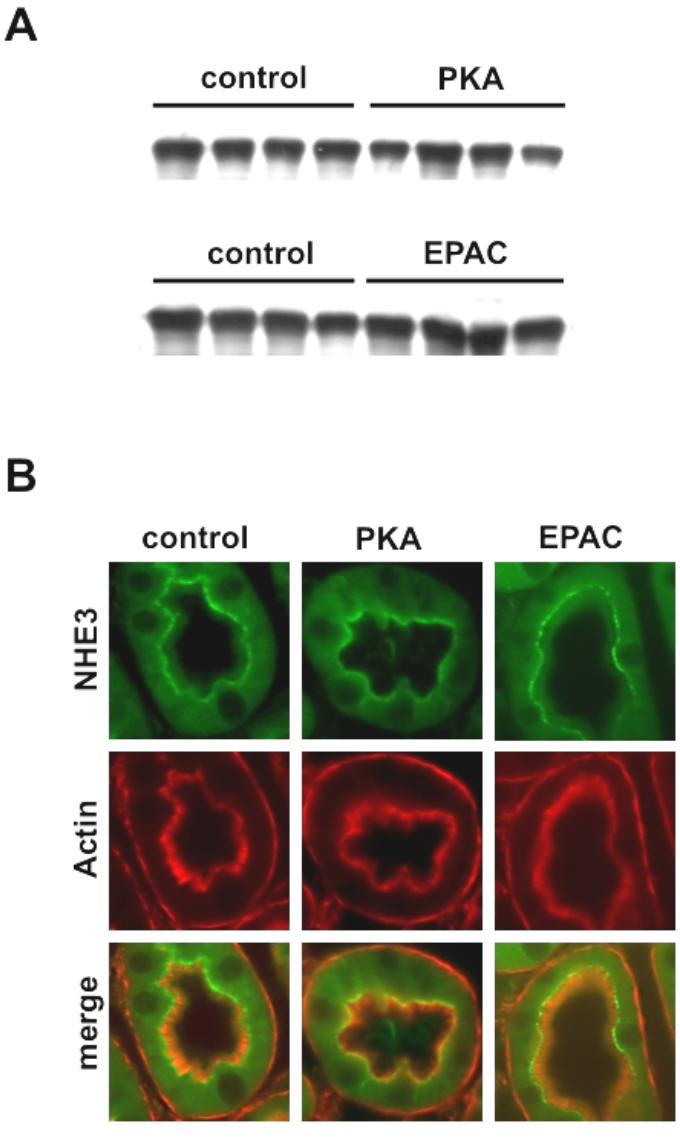

Figure 25: **Expression of NHE3 in mouse BBM**

Kidney slices were incubated for 30 min with the indicated analogs (50 μM). **A)** Western blot of BBM with an anti-NHE3 antibody: BBM isolated from 4 independent experiments were processed in parallel. **B)** Immunofluorescence of kidney slices with anti-NHE3 antibody (green) as well as with phalloidin for actin detection (red).

6. 8 NHE3 phosphorylation

OK cells were phosphorylated and NHE3 was immunprecipitated as indicated in material and methods. Fig. 26A shows an autoradiogram of a representative experiment. Fig. 26B represents the average of 6 independent experiments. This data indicated that PKA phosphorylated NHE3, while EPAC had no effect.

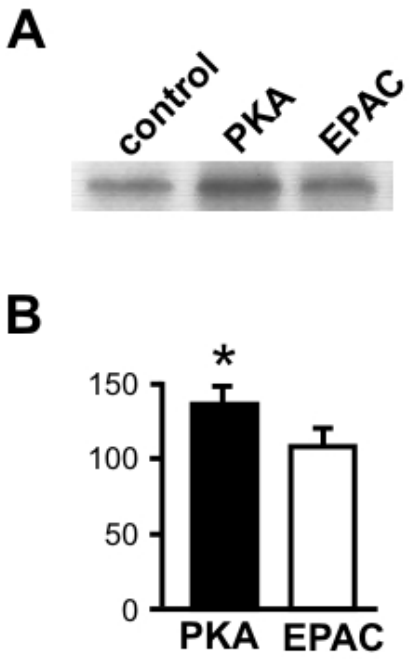

Figure 26: NHE3 phosphorylation

A) Autoradiogram of ^{32}P-phosphorylation of NHE3. **B)** Summary of 6 independent experiments.

6. 9 Effect of cAMP analogs on NaPi-IIa activity

To study the contribution of the PKA- and EPAC-dependent pathways in the regulation of NaPi-IIa, we performed ^{32}P-uptakes in OK cells treated for 4 h in the presence of several concentrations ($5x10^{-4}$, 10^{-4}, $5x10^{-5}$, 10^{-5}, $5x10^{-6}$ M) of the cAMP analogs. As a control for NaPi-IIa downregulation, cells were also incubated with 10^{-8} M 1-34 PTH. As shown in Fig. 27, the Na^+-dependent ^{32}P-uptake was reduced to about 40% of the control value upon incubation of the cells with PTH. Furthermore, the ^{32}P-uptake was also inhibited in a concentration-dependent manner by 8-Br-cAMP as well as by the PKA activator. At the higher concentration ($5x10^{-4}$ M), both analogs induced a percentage of inhibition similar to that induced by PTH. The Na^+-dependent ^{32}P-uptake was otherwise not affected by incubation of OK cells

with the EPAC activator (Fig. 27). Furthermore, incubation with sub-maximal concentrations of the PKA together with the EPAC activator did not result in a stimulation of the former one (data not shown).

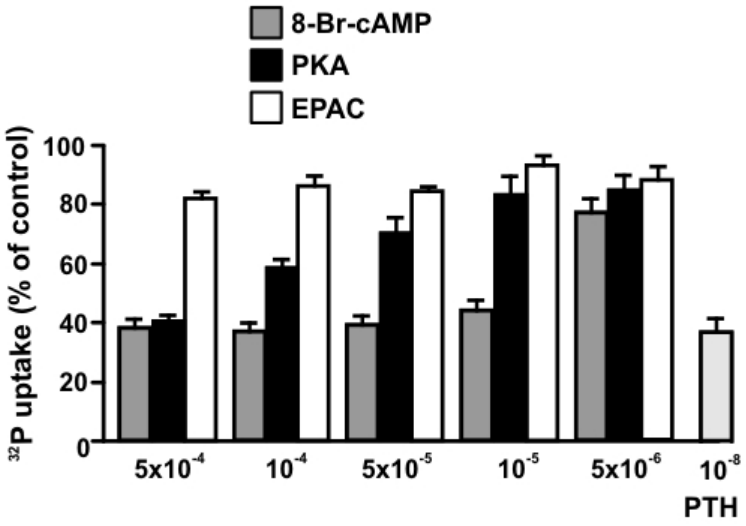

Figure 27: Activity of NaPi-IIa

Na^+-dependent ^{32}P-uptake: cells were incubated for 4 h in the absence or presence of 10 nM 1-34 PTH or the indicated concentrations of the cAMP analogs.

6. 10 Effect of cAMP analogs on the pattern of NaPi-IIa expression

The pattern of expression of NaPi-IIa in proximal tubules was analyzed upon incubation with the different cAMP analogs. For that purpose, kidney slices were incubated with a NaPi-IIa antibody as well as with phalloidin. The first row of Fig. 28 shows overviews of kidney sections stained with the NaPi-IIa antibody. Rows 2-4 show higher magnifications of proximal tubules, where the signal corresponding to NaPi-IIa is shown in green and the actin signal in red. In untreated samples NaPi-IIa was concentrated in the BBM of proximal tubules. The colocalization of NaPi-IIa with actin within the BBM is indicated by the yellow color of the merged picture. Incubation with either 8-Br-cAMP (second column) or the PKA activator (third column) induced internalization of NaPi-IIa, as indicated by the reduction of the immunosignal in BBM. On the contrary, the pattern of expression of NaPi-IIa was not affected by the EPAC activator (forth column).

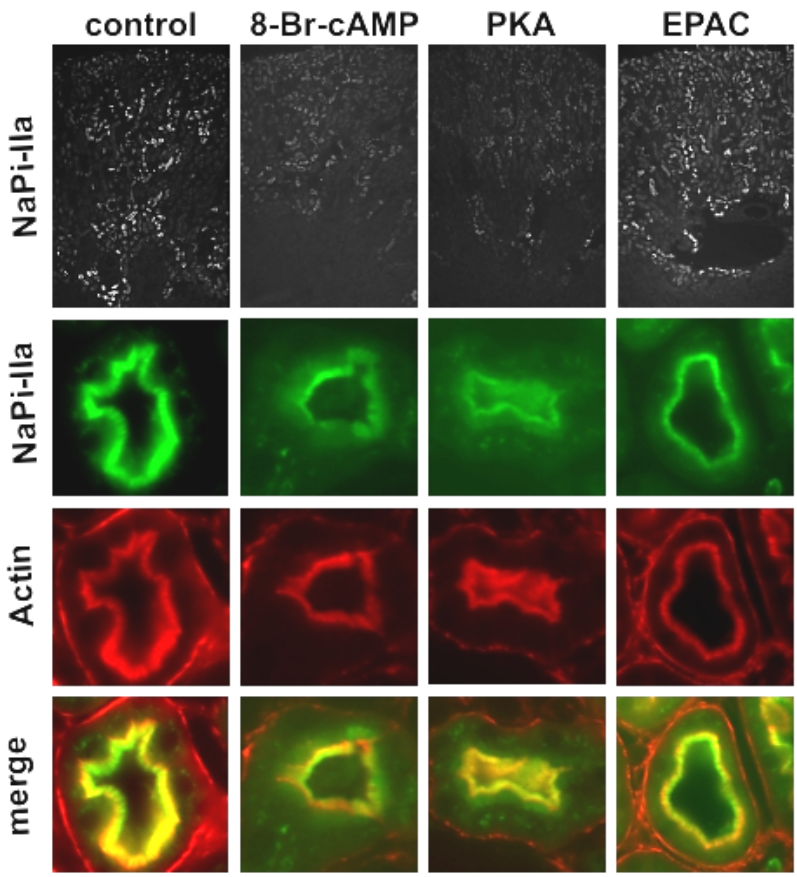

Figure 28: Expression of NaPi-IIa

Kidney slices were incubated in the absence or presence of 50 µM of the indicated analogs and processed for immunofluorescence with anti-NaPi-IIa antibody (green) and phalloidin (red). The first row shows overviews of kidney sections, rows 2-4 show higher magnifications of proximal tubules.

7. Discussion

The Na/H exchanger 3 (NHE3) and the type IIa Na/Pi cotransporter (NaPi-IIa) are two Na^+-dependent transporters expressed in the brush border membrane (BBM) of renal proximal tubules. These transporters contribute to a large extent to pH and P_i homeostasis. Their activities are regulated by many hormonal and non-hormonal factors. In particular, the activities of both transporters are downregulated upon increases in the intracellular levels of cAMP induced by different hormones, such as parathyroid hormone (PTH) or dopamine (DA). Such regulation is mediated, at least partially, by activation of protein kinase A (PKA). However, the potential implication of EPAC, a guanine nucleotide exchange protein directly activated by cAMP, in the regulation of NHE3 and NaPi-IIa had not been investigated. Therefore, the aim of this work was to study whether or not EPAC plays a role in the cAMP-induced downregulation of NHE3 and NaPi-IIa.

7. 1 EPAC1 expression in the BBM of murine proximal tubules

Two isoforms of EPAC have been described so far: EPAC1 and EPAC2 (Kawasaki, Springett et al. 1998; Bos 2003). Both isoforms bind cAMP and have GEF (guanine nucleotide exchange factor) activity towards the small GTPases Rap1 and Rap2 (de Rooij, Zwartkruis et al. 1998). Consequently, EPAC1 and EPAC2 mediate the cAMP-dependent exchange of GTP for GDP that results in activation of Rap1/2. However, both EPAC isoforms differ in their primary structure, affinity for cAMP and tissue distribution (Kawasaki, Springett et al. 1998; Laroche-Joubert, Marsy et al. 2002).

EPAC1 shows a broader pattern of expression and its mRNA has been detected in several nephron segments (Kawasaki, Springett et al. 1998; Laroche-Joubert, Marsy et al. 2002). In contrast, EPAC2 seems to have a very restricted tissue distribution and its mRNA has been detected only in some regions of the brain and adrenal glands (Kawasaki, Springett et al. 1998). RT-PCR on microdissected nephron segments of rat has shown mRNA expression of EPAC1 and Rap1 along the whole nephron. The highest expression of EPAC1 mRNA was found in glomeruli, proximal convoluted tubules, cortical collecting duct and outer and inner medullary collecting duct (Laroche-Joubert, Marsy et al. 2002). However, renal expression of EPAC1 at the protein level had not been analyzed. Therefore, the expression of EPAC1 protein in mouse kidney was first studied. For that purpose, mouse kidney slices were processed for immunohistochemistry using an antibody directed against the C-terminus of EPAC1. EPAC1 was detected in all segments of proximal tubules, although the strongest staining was found in S2 and the weakest in S3 segments. In all segments, EPAC1 was concentrated at the BBM. Glomeruli and distal parts of the nephron showed no EPAC1 signal. The discrepancy between the pattern of expression of EPAC1 mRNA reported previously (Laroche-Joubert, Marsy et al. 2002) and protein

expression described in this study may be due to either species-related differences (rat versus mouse) or may reflect actual differences between mRNA and protein expression.

Immunostainings of consecutive kidney sections with the corresponding antibodies indicated that EPAC1, NHE3 and NaPi-IIa share similar patterns of expression within the BBM of proximal tubules. This common location validated the study of a potential role of EPAC1 in the regulation of both transporters.

7. 2 Use of modified cAMP analogs to activate preferentially PKA or EPAC

Modified analogs of cAMP, such as 8-Br-cAMP, activate EPAC and PKA equally well as cAMP (Enserink, Christensen et al. 2002; Christensen, Selheim et al. 2003). However, analogs modified in the 6'-position of the ribose (6-MB-cAMP) are poor EPAC activators and full PKA activators compared to cAMP. In contrast, analogs modified in the 2'-position (8-pCPT-2'-O-Me-cAMP) induce stronger EPAC activation than cAMP but are only partial agonists for PKA (Enserink, Christensen et al. 2002; Christensen, Selheim et al. 2003).

In this study, the effects of 8-Br-cAMP (EPAC and PKA activator), 8-pCPT-2'-O-Me-cAMP (EPAC activator) and 6-MB-cAMP (PKA I activator) on NHE3 and NaPi-IIa activity were compared. Studies were performed in two different systems: Opossum kidney (OK) cells and slices from mouse kidney cortex. OK cells represent a well characterized cellular model for renal proximal epithelia. These cells express endogenous NHE3 and NaPi-IIa transporters. Both transporters are properly expressed in the BBM of this particular cell model and are regulated by the same factors that regulate their activity in kidney. However, this model may have some limitations, since, as we have previously reported, OK cells do not express PDZK1, a protein located in the BBM of mouse proximal tubules known to interact with NaPi-IIa (Gisler, Stagljar et al. 2001; Hernando, Deliot et al. 2002). On the other hand, our lab has shown that slices from mouse kidney cortex retain their morphological and functional properties within the time frame in which the effect of the different analogs was investigated (Bacic, Schulz et al. 2003). However, we can not rule out partial protein degradation in the transcurs of incubation. NHE3 activity was determined in OK cells by measurements of Na^+-dependent intracellular pH recovery rates, and in BBMV (isolated from slices of mouse kidney cortex) by acridine-orange fluorimetry; NaPi-IIa activity was determined in OK cells by measurements of Na^+-dependent ^{32}P-uptake.

7. 3 NHE3 activity is inhibited via both the PKA and the EPAC pathway

The Na^+-dependent recovery rate of intracellular pH in OK cells reflects the activity of the endogenous NHE3, as this renal proximal cell line expresses specifically this NHE isoform (Azarani, Goltzman et al. 1995). The recovery rate after the first acidification was taken as 100% for every individual experiment. This recovery rate was reduced by about 50% after 15 min incubation of the OK cells with 50 μM 8-Br-cAMP. A similar reduction was observed after incubation of OK cells with 50 μM of the PKA and EPAC activators. Therefore, specific activation of PKA or EPAC led to inhibition of NHE3 in OK cells.

PKA and EPAC activators also inhibited NHE activity in BBMV isolated from slices of mouse kidney cortex. Incubation with PKA and EPAC activators induced a concentration-dependent inhibition of NHE activity. These results demonstrated that the PKA and EPAC activators are able to inhibit NHE3, suggesting that cAMP-induced inhibition of NHE3 may be mediated via both pathways.

A Specificity of PKA and EPAC effects

Available cAMP analogs preferentially, but not exclusively, activate PKA or EPAC (Enserink, Christensen et al. 2002; Christensen, Selheim et al. 2003). In both cases, the use of concentrations above a threshold value leads to cross-activation of both cascades. Therefore, experiments were run in order to rule out cross-activation of PKA upon incubation of kidney samples with the EPAC activator. For this purpose, two parallel experiments were performed: a) direct determination of enzymatic PKA activity, by using an exogenous biotin-labelled PKA substrate; b) indirect determination of PKA activity, by performing Western blots with a PKA substrate antibody.

Homogenates from slices treated with each activator were incubated with a biotinylated PKA-substrate in the presence of γ^{32}P-ATP. Upon incubation, the substrate was purified from the reaction mix using streptavidin-coated filters and the incorporation of ^{32}P was counted. Homogenates from kidney slices incubated with 50 μM of the PKA activator led to an increase in phosphorylation of the biotinylated PKA substrate, as compared to non-treated samples. However, homogenates derived from slices incubated with the EPAC activator did not induce phosphorylation of the exogenous PKA substrate over basal levels. Preincubation with a PKA inhibitor (H89) reduced the basal PKA activity in homogenates and partially prevented the phosphorylation promoted by the PKA activator. These results showed that incubation with the PKA, but not with the EPAC activator resulted in the enzymatic activation of PKA. In agreement with this data, Western blots with an anti-PKA substrate antibody indicated that incubation of BBMV with the PKA activator led to phosphorylation of several PKA substrates, whereas incubation with the EPAC activator did not result in such PKA-dependent phosphorylation. Taking these data together, they strongly suggest that incubation of kidney slices with 50 μM of the EPAC activator did not result in cross-activation of PKA, and therefore its effect on NHE3 activity is specific.

It has been previously reported that the PKA inhibitor H89 fully or partially prevented the NHE inhibition induced by 8-Br-cAMP or factors that increase intracellular cAMP levels (Felder, Campbell et al. 1990; Azarani, Goltzman et al. 1995; Di Sole, Casavola et al. 1999; Wiederkehr, Di Sole et al. 2001; Pedrosa, Gomes et al. 2004). Therefore, the effect of the PKA inhibitor on the PKA- and EPAC-dependent NHE inhibition was analyzed by preincubating kidney slices with 100 μM H89 prior to addition of the cAMP analogs. As expected, preincubation of kidney slices with H89 fully abolished the NHE inhibitory effect induced by 50 μM PKA activator. However, the inhibition generated by 50 μM EPAC activator was similar in the absence or presence of the PKA inhibitor. This finding, together with the PKA activation data discussed above, indicated that the effect of EPAC on NHE activity was specific and independent of PKA. Thus, the previously reported observations, where H89 blocked the inhibition generated by 8-Br-cAMP, probably reflected the reduced ability of this analog to activate EPAC, as compared to 8-pCPT-2'-O-Me-cAMP. However, it should be noted that the specificity of the cAMP analogs with regard to PKA activation was lost upon incubation at higher concentrations. Thus, at 100 μM both analogs stimulated PKA activity in kidney homogenates and in both cases the inhibitory effect on NHE was partially prevented by H89.

B PKA and EPAC effects are not additive

PKA and EPAC may regulate cAMP signaling either synergistically or in an opposite way. Thus, in HEK cells, PKB was activated upon transfection with EPAC, whereas stimulation of PKA inhibited PKB activity (Mei, Qiao et al. 2002). These opposite effects of EPAC and PKA may provide a molecular mechanism for the cell-specific effects of cAMP. In contrast, PKA and EPAC acted synergistically to promote neurite extensions in PC-12 cells (Christensen, Selheim et al. 2003). To analyze whether PKA and EPAC have a synergistic effect on the regulation of NHE3, kidney slices were incubated either with each activator alone or with both activators together. All three treatments led to similar percentages of NHE3 inhibition. Therefore, simultaneous activation of both pathways did not have an additive effect on NHE3 inhibition, suggesting that PKA and EPAC may compete for common downstream effectors.

C Downstream effectors

As indicated above, EPAC1 (and EPAC2) mediates the cAMP-dependent exchange of GTP for GDP that activates Rap1/2 (de Rooij, Zwartkruis et al. 1998). Although the precise downstream signal(s) that connect(s) EPAC activation with the cellular response is not known, several intracellular cascades have been proposed. Thus, activation of PLC-ε, PKB and ERK1/2 may mediate some effects, yet the emerging picture suggests that the pathway is cell type specific.

PLC-ε is a recently identified isoform of the phospholipase C (PLC) family that is regulated by heterotrimeric G-proteins as well as by monomeric GTPases (Kelley, Reks et al. 2001; Schmidt, Evellin et al. 2001; Evellin, Nolte et al. 2002). Thus, PLC-ε is activated by Gβγ-dimers released upon activation of G-protein coupled receptors and by $G\alpha_{12/13}$- (but not $G\alpha_q$) and $G\alpha_s$-coupled receptors. $G\alpha_s$-induced activation of PLC-ε is mediated by EPAC and involves activation of the Rap2B GTPase. Indeed, GTP-dependent binding of Rap2B to the PLC-ε Ras-binding domain has been demonstrated (Schmidt, Evellin et al. 2001; Evellin, Nolte et al. 2002). We studied the potential implication of PLCs in the PKA- and EPAC-induced downregulation of NHE3. The PLC activity of BBM treated with different analogs was measured indirectly using a fluorescent-based technique. No changes in total PLC activity were detected upon incubation with 8-Br-cAMP or with the PKA activator whereas the EPAC activator induced a moderate (but statistically non-significant) increase in PLC activity. Therefore, the different cAMP analogs do not seem to induce major changes in PLC activity.

PKB could be a common effector for PKA and EPAC. Direct activation of PKB by PKA has been reported (Filippa, Sable et al. 1999). This activation is independent of the classical PI3K pathway but requires the threonine 308 residue of PKB, suggesting that PKA may lead to phosphorylation of PKB. This pathway, however, does not seem to be universal, since in some cells PKA inhibits, rather than activates, PKB (Kim, Jee et al. 2001). This inhibition requires PKA phosphorylation of Rap1b and seems to involve the blockage of the coupling between PKB and its upstream activator PDK. On another hand, activation of EPAC has been shown to lead to PKB activation, also in a Rap1-dependent fashion. In this case, PKB activation seems to relay on the classical PI3K pathway. In order to study the potential implication of PKB in the PKA- and EPAC-dependent inhibition of NHE3, we analyzed kidney cortex homogenates for changes in the pattern of expression of phosphorylated PKB substrates. Homogenates were processed for Western blots using a phospho-(serine/threonine)-PKB substrate antibody. Several phosphorylated PKB substrates were detected under basal conditions. The two most prominent substrates had an apparent molecular weight of 42 and 100 KDa, respectively. However, no significant changes in the phosphorylation pattern were detected upon incubation of kidney slices with the different cAMP analogs. Although this data is not a final probe, it does strongly suggest that PKB signaling is not involved in the PKA- and EPAC-induced inhibition of NHE3 in kidney.

As PKB, ERK1/2 may represent a common effector for PKA and EPAC. ERK signaling couples extracellular signals to cellular effects through the GTPase Ras. Active Ras binds and activates Raf-1; activated Raf-1 phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. Activation of MEK1/2 (and downstream kinases ERK1/2) is responsible for cAMP stimulation of H^+, K^+-ATPase in kidney cells, an effect attributed to EPAC (Laroche-Joubert, Marsy et al. 2002). However, the functionality of EPAC in ERK signaling remains in contention, because in many cell lines EPAC (stimulated both directly and via GPCR) had no impact on ERK signaling (Enserink, Christensen et al. 2002). To study whether or not the MEK1/2-ERK1/2 signaling pathway plays a role in the PKA- and EPAC-induced downregulation of NHE3, the effects of both analogs were investigated in the absence/presence of the MEK1/2 inhibitor PD098059. Incubation of kidney slices with PD098059 partially prevented the PKA effect, whereas it fully blocked the inhibition generated by the EPAC

activator. This data suggested that MEK1/2 is a common effector of the PKA and EPAC pathways with regard to NHE3 inhibition. However, PKA must also signal through additional intracellular cascades to achieve a full effect, since the MEK1/2 inhibitor did not fully prevent the PKA-induced inhibition of NHE3. We could not observe changes in the phosphorylation state of ERK1/2 upon activation of either pathway. As MEK family members are considered among the most selective kinases, further experiments are required to reconcile the full or partial inhibition generated by the MEK1/2 inhibitor with the lack of ERK1/2 phosphorylation.

D NHE3 inhibition is not mediated by endocytosis of the exchanger

To study whether the inhibition of NHE3 generated by the different cAMP analogs in OK cells was related to changes in the total amount of the transporter, cell lysates were processed for Western blot using a polyclonal antibody raised against full length NHE3. Quantification of three independent experiments revealed similar amounts of NHE3 in all experimental conditions. This result suggested that in OK cells, the inhibition of NHE3 activity induced by all cAMP analogs took place without changes in total amount of protein.

In order to study whether the inhibition was due to changes in the amount of surface-expressed NHE3, plasma membrane proteins were labelled by incubating OK cells with biotin. Upon biotinylation, labelled proteins were immunoprecipitated with streptavidine and processed for Western blot using the NHE3 antibody described above. Quantification of three independent experiments indicated that the amount of NHE3 expressed in the plasma membrane was similar in control and treated cells. Therefore, these surface biotinylation experiments showed that inhibition of NHE3 was not mediated by a reduction of surface-expressed transporter. As previously reported (Collazo, Fan et al. 2000), a reduction in surface expression of NHE3 was observed after 4 h incubation in the presence of PTH, indicating that the biotinylation assay is sensitive enough to detect changes in plasma membrane-bound NHE3. The above findings are in agreement with previous reports showing that in OK cells, acute PTH (Collazo, Fan et al. 2000) or DA treatment (Wiederkehr, Di Sole et al. 2001) first inhibited NHE3 activity without changing its membrane expression. Only after longer exposure, PTH-induced inhibition also involved dynamin-dependent endocytosis, suggesting retrieval of the exchanger via clathrin-coated pits (Collazo, Fan et al. 2000).

To study whether the PKA- and EPAC-induced inhibition of NHE in kidney was due to changes in the amount of exchanger, kidney slices were processed for Western blots and immunostaining with anti-NHE3 antibody. Western blots of BBM indicated that PKA and EPAC stimulation did not change the total amount of NHE3. Furthermore, the expression pattern of the exchanger in BBM remained unaffected upon incubation with different agonists. These results suggested that, as in OK cells, inhibition of NHE3 was not mediated by a reduction of NHE3 in the BBM. This finding is in agreement with previous reports showing that acute PTH induced inhibition of NHE3 did not involve endocytosis of

the exchanger in rat proximal tubules (Fan, Wiederkehr et al. 1999; Yang, Maunsbach et al. 2004). Thus, exposure to PTH for 1 hour led to a redistribution of NHE3 from the tips to the base of the proximal tubule microvilli; however, NHE3 was never detected in AP-2 or horseradish peroxidase positive compartments, indicating the absence of endocytosis (Yang, Maunsbach et al. 2004). Moreover, in parathyroidectomized rats, acute intravenous bolus of PTH first inhibited NHE3 in the absence of changes on BBM expression, whereas a decrease in surface-expression was observed only 4-12 h after the PTH bolus (Fan, Wiederkehr et al. 1999).

Our current understanding regarding the molecular mechanism that underlies cAMP-induced inhibition of NHE3 in the absence of changes in the amount of transporter expressed at the BBM is mostly based on results obtained with cellular models (OK cells as well as fibroblast transfected with different combinations of NHE3 and regulatory proteins). Thus, in its PKA-depending component, this inhibition is due to PKA-mediated phosphorylation of NHE3 on several serine residues. In turn, phosphorylation behaves as a signal to trigger the late on-set clathrin-mediated endocytosis of NHE3. A central component of this regulatory process is the scaffolding protein NHERF1 (Shenolikar and Weinman 2001). NHERF1 is expressed in the apical membrane of different segments of the nephron, and colocalizes with NHE3 in the BBM of proximal tubules. NHERF1 contains two PDZ (PSD95/DglA/ZO-1) domains, which are modules of about 90 amino acids involved in protein-protein interaction. NHE3 has been shown to bind to the PDZ-2 (most C-terminal) domain. In addition, the C-terminal tail of NHERF1 contains a MERM (Merlin-Ezrin-Radixin-Moesin)-binding domain that mediates binding to cytoskeletal proteins of the MERM family. This MERM-binding domain has a dual role. On one hand it determines the apical location of NHERF1; on another hand it connects NHERF1 with PKA through Ezrin, since Ezrin has been shown to have AKAP (A-kinase anchor protein) activity (Weinman, Steplock et al. 2000). cAMP-induced downregulation of NHE3 requires the presence of NHERF1, and both the PDZ-2 domain as well as the MERM-binding domain are involved in this process. Thus, NHERF1 behaves as a bridge that brings NHE3 and PKA into proximity, allowing the PKA-mediated phosphorylation of NHE3 that inactivates the transporter (Moe, Amemiya et al. 1995).

E NHE3 phosphorylation by PKA, but not EPAC

In OK cells, PTH-induced inhibition of NHE3 activity has been shown to proceed in parallel with phosphorylation of the transporter (Collazo, Fan et al. 2000). Moreover, this PTH-dependent phosphorylation was prevented by PKA inhibitors. Therefore, we next studied the effect of the PKA- and EPAC-activating analogs on the state of phosphorylation of NHE3 in OK cells. We found that NHE3 is constitutively phosphorylated. Collazo *et al* have previously shown that this basal phosphorylation takes place mostly on serine residues (Collazo, Fan et al. 2000). Incubation with the PKA-activating analog induced an increase in phosphorylation, whereas activation of EPAC had no effect. These results suggest that unlike the PKA effect, the EPAC-induced inhibition of NHE3 is independent of phosphorylation of the transporter.

7. 4 Inhibition of NaPi-IIa activity via PKA, but not EPAC

As for NHE3, cAMP-induced downregulation of NaPi-IIa is well documented. Most studies have been done in the context of PTH signaling and suggest a preferential implication of cAMP upon activation of basolateral PTH receptors (Traebert, Volkl et al. 2000). With few exceptions, information gathered so far suggests that PTH inhibits NaPi-IIa by promoting endocytosis and degradation of the cotransporter (Lotscher, Kaissling et al. 1996). Recently, our group has shown that activation of apical, but not basolateral, D1-like dopamine receptors induce NaPi-IIa internalization, by a mechanism dependent on PKA, but independent of PKC (Bacic, Capuano et al. 2005).

To study the contribution of the PKA- and EPAC-dependent pathways in the regulation of NaPi-IIa, we performed ^{32}P-uptakes in OK cells treated for 4 h in the presence of several concentrations of cAMP analogs. OK cells are known to express a NaPi-IIa cotransporter controlled by the major factors regulating the cotransporter in the kidney (Pfister, Lederer et al. 1997; Pfister, Hilfiker et al. 1998). The Na^+-dependent ^{32}P-uptake was inhibited in a concentration-dependent manner by 8-Br-cAMP as well as by the PKA activator. The highest tested concentration of both analogs ($5x10^{-4}$ M) induced a reduction in uptake similar to that induced by 10^{-8} M PTH; this concentration of PTH is known to lead to maximal inhibition of NaPi-IIa in OK cells. However, the Na^+-dependent ^{32}P-uptake was not affected upon incubation of OK cells with EPAC activator. Furthermore, incubation with sub-maximal concentrations of the PKA activator together with the EPAC activator did not result in a stimulation of the former one. These findings suggest that cAMP inhibits NaPi-IIa by activating the PKA-dependent pathway, whereas the EPAC-dependent signaling is not involved in this process. Our group and others have previously reported that PTH- and DA-induced inhibition of NaPi-IIa occurs as a consequence of membrane retrieval, followed by lysosomal degradation of the cotransporter (Lotscher, Kaissling et al. 1996; Traebert, Volkl et al. 2000; Yang, Maunsbach et al. 2004; Bacic, Capuano et al. 2005). Therefore, we analyzed the pattern of expression of NaPi-IIa in kidney slices upon incubation with different cAMP analogs. Incubation with 8-Br-cAMP or PKA activator induced internalization of NaPi-IIa, indicated by reduction of the immunosignal in BBM, whereas the EPAC activator had no effect. Therefore, these results suggest that cAMP-induced downregulation of NaPi-IIa involves the PKA-, but not the EPAC-dependent pathway.

In summary, this study shows that EPAC1 is expressed in the BBM of renal proximal tubules where it colocalizes with NHE3 and NaPi-IIa. Activation of both EPAC1 and the classical PKA pathway mediate the cAMP-induced inhibition of NHE3. However, PKA but not EPAC, mediates the cAMP-dependent inhibition of NaPi-IIa. For an overview see Fig. 29.

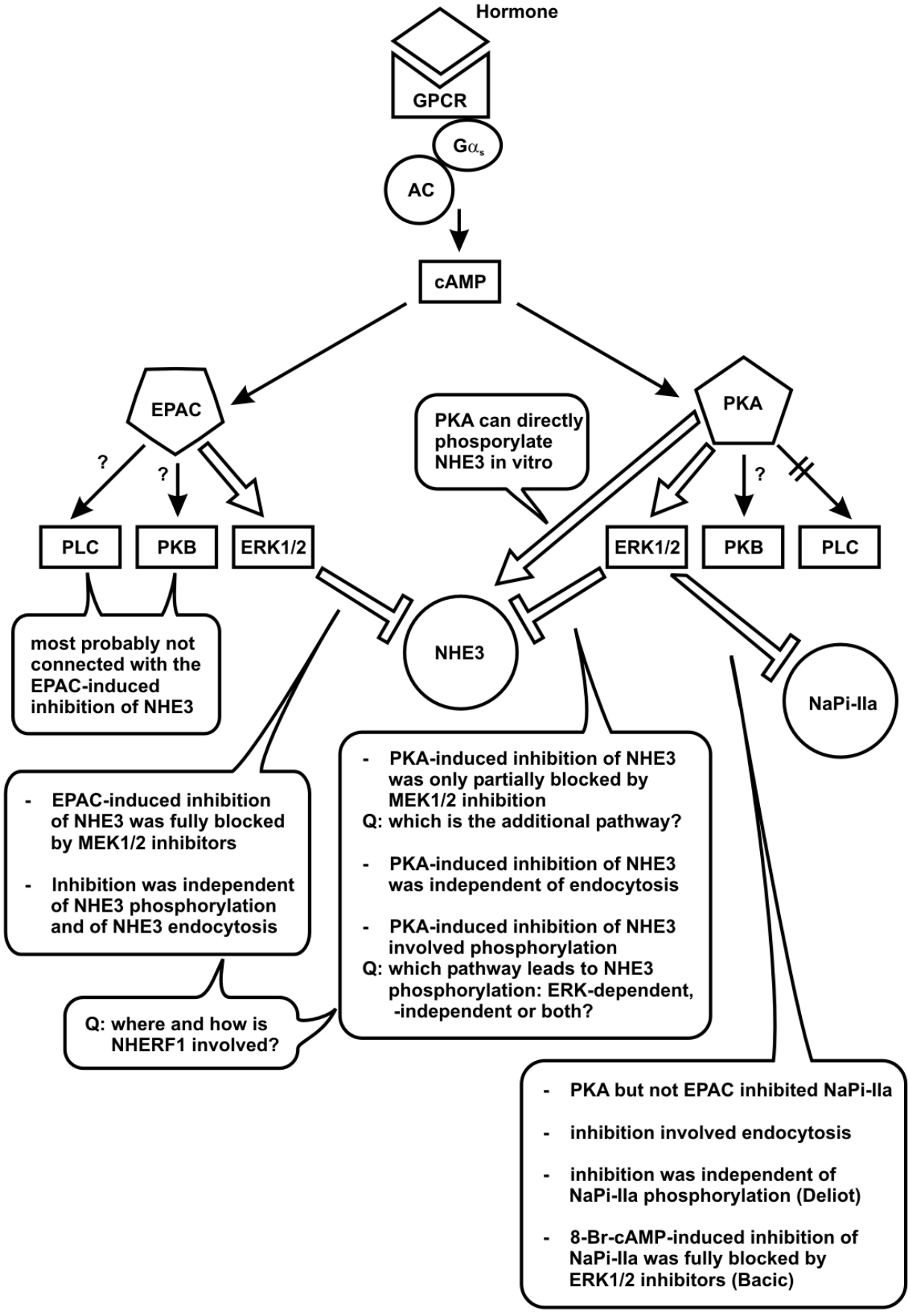

Figure 29: Overview

8. Future perspectives

8. 1 Regulation of NHE3

In this study we found that stimulation of both PKA and EPAC inhibited the activity of NHE3 without acute changes in the surface expression of the exchanger. The PKA-induced inhibition of NHE3 was partially sensitive to disruption of the MEK1/2-ERK1/2 kinase pathway whereas the EPAC-induced response was completely prevented upon inhibition of these kinases. The PKA, but not the EPAC activator, induced phosphorylation of the exchanger. Therefore, further studies should be performed in order to:

a) Study which steps connect the activation of ERK1/2 to the inhibition of NHE3.

b) Identify which other intracellular cascades, in addition to ERK1/2, are responsible for the PKA-specific response. By using the kidney slices assay, the sensitivity of the PKA-induced response to inhibitors of intracellular cascades should be tested. In particular, the implication of the PKB pathway should be readdressed using this assay.

c) Study which casacade (ERK-dependent or ERK-independent) is linked to the PKA-induced phosphorylation of NHE3. For that purpose phosphorylation of NHE3 upon treatment with the PKA activator should be studied in the absence/presence of ERK inhibitors.

d) Analyze whether the PKA-induced phosphorylation of NHE3 involves serine 552 and/or serine 605. Previous studies have identified these amino acids as the sites phosphorylated upon treatment of cells with 8-Br-cAMP (Kurashima, Yu et al. 1997; Zhao, Wiederkehr et al. 1999). Single or double mutants containing these residues replaced by alanine and glycine, respectively, showed not or reduced regulation by PKA (Zhao, Wiederkehr et al. 1999). AP-1 cells (devoid of NHE3) should be transfected with serine 552 and/or serine 605 mutants. Then, the effect of PKA on the state of phosphorylation of the mutated NHE3 will be analyzed.

e) Analyze whether serine 634 is required for both the PKA- and EPAC-induced inhibitions. Serine 634 has been reported to be essential for cAMP downregulation even though this residue does not become phosphorylated upon cAMP treatment (Zhao, Wiederkehr et al. 1999). Therefore, AP-1 cells (devoid of NHE3) could be transfected with serine 605-mutated NHE3 and the effect of PKA and EPAC compared.

f) Study the contribution of the Na/H-exchanger regulatory factor 1 (NHERF1) on the PKA and EPAC-dependent inhibition of NHE3. The cAMP-dependent inhibition of NHE3 has been shown to depend on NHERF1 expression (Weinman, Steplock et al. 2003). NHERF1 has been proposed to connect PKA with NHE3 due to its ability to bind NHE3 directly and PKA indirectly (via Ezrin). Kidney samples derived from NHERF1 -/- mice should be treated with PKA and EPAC activators and their effect on NHE3 activity compared.

8. 2 Regulation of NaPi-IIa

This study also shows that, in contrast to NHE3, cAMP regulation of NaPi-IIa involves activation of PKA but not of EPAC, and that this inhibition is due to endocytosis and degradation of the protein. Therefore, further studies should be undertaken in order to:

a) Dissect the steps connecting ERK1/2 activation to NaPi-IIa endocytosis. We have shown previously that inhibition of NaPi-IIa upon stimulation of both apical and basolateral PTH receptors is mediated, at least partially, by activation of ERK1/2 (Bacic, Schulz et al. 2003). In addition, we have also shown that this inhibition is not mediated by phosphorylation of the cotransporter (Deliot, Hernando et al. 2005).

b) Study the dependency of the PKA-induced inhibition of NaPi-IIa on proteins known to interact with the cotransporter. In this regard, we have shown that NaPi-IIa interacts with D-AKAP2, a dual PKA anchoring protein (Gisler, Stagljar et al. 2001).

c) Identify which other intracellular cascades, in addition to ERK1/2, are responsible for the PTH-induced endocytosis of the cotransporter. We previously reported that, while endocytosis and degradation of NaPi-IIa upon PKA stimulation is fully blocked by ERK1/2 inhibitors, the response to PKC is only partially prevented upon ERK1/2 inhibition (Bacic, Schulz et al. 2003).

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